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TABLE OF CONTENTS

Cover	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	20
Reportable Outcomes.....	21
Conclusions.....	22
References.....	22
Appendices	
Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Dai, D., Goldstein, J. A. and Hodgson, E. (2001) Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse and rat liver. microsomes. Drug Metabol. Disp. 29:12-1-1204.....	25
Dai, D., Tang J., Rose, R. L., Hodgson, E., Bienstock, R. J., Mohrenweiser, H. W. and Goldstein, J. A. (2001). Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. J. Pharnacol. Exptl. Therap. 299:825-831.....	29
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INTRODUCTION

Chemicals used during deployments, and particularly interactions between such chemicals, are frequently cited as possible causative agents in deployment-related illnesses. Unfortunately, definitive evidence for or against such a role is lacking. In part this is related to the fact that investigations of deployment-related chemicals carried out in experimental animals assume relevance only when they can be extrapolated with confidence to humans. Since most exogenous chemicals can be substrates, inhibitors and/or inducers of xenobiotic-metabolizing enzymes (XMEs), these enzymes are an important potential locus for interactions. Many XMEs are polymorphic, potentially putting some individuals at greater risk than others. We are examining the role of specific human XMEs on the metabolism of a subset of chemicals important in military deployments. Chlorpyrifos, diethyl toluamide (DEET), permethrin, pyridostigmine bromide, sulfur mustard and its degradation products are being tested as substrates and inhibitors of the most important human XMEs, including cytochrome P450s (CYPs), flavin-containing monooxygenases (FMOs), alcohol and aldehyde dehydrogenases and esterases. The potential of the test chemicals to act as XME inducers is also being tested in mice. HPLC methods have been either adapted or developed to facilitate these studies. The results will enable findings from animal studies to be extrapolated to humans with confidence, permit the identification of populations at risk and provide analytical methods for exposure assessment.

BODY OF REPORT

The five items outlined in the Statement of Work for Year 2 have been accomplished and additional methods development needed to facilitate these studies have been carried out.

Part 1. *"Further development of new HPLC methods or validation and/or modification of existing methods for analysis of all test compounds and their metabolites, now including a method for analysis of an endogenous substrate, testosterone, potentially involved in interactions.*

Chlorpyrifos:

The method developed for the analysis of chlorpyrifos and its metabolites was described, in detail, in the last annual report. This method has proven most useful, resulting in two peer-reviewed papers (Tang et al., 2001 and Dai et al., 2001 - copies attached) and is currently in use in ongoing studies.

Chlorpyrifos studies were also supported, in part, by the North Carolina Environmental Trust Fund.

DEET:

The method developed for the analysis of DEET and its metabolites (*N*-ethyl-*m*-toluamide (ET) and *N*, *N*-diethyl-*m*-hydroxymethylbenzamide (BALC) utilized the same Shimadzu HPLC

system (Kyoto, Japan), and the same column, as that used for chlorpyrifos and was described in detail in the last annual report.

Permethrin:

The preliminary studies of a method for analysis of permethrin and its metabolites described in the last annual report have been extended (see results under tasks 2 - 4, below) and will be submitted as a separate manuscript to a peer-reviewed analytical methods journal.

Pyridostigmine bromide:

A reverse phase high pressure liquid chromatography (HPLC) method was developed for the separation and detection of pyridostigmine bromide and its potential metabolites based on the HPLC method of Leo (1997). The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of 2 pumps (LC-10AT VP) a Shimadzu auto injector (SIL-10AD VP), and a Shimadzu UV/VIS detector (SPD-10A VP). All system components were controlled through the Shimadzu powerline firmware. Data was collected via a Shimadzu system controller (SCL-10A VP) and analyzed using CLASS-VP 7.0 software. The mobile phase consisted of 5% methanol, 5% acetonitrile, 0.1% triethylamine, and 89.9% ammonium acetate buffer, pH 4.0 (adjusted with acetic acid) running at 1.0 ml/min. Metabolites were separated by a Synergi Max column (Synergi 4 μ , 150 x 4.60 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 260 nm.

Sulfur Mustard and its Degradation Products:

These compounds have always presented an analytical challenge. Their water solubility and their lack of suitable chromophores in the UV / visible region make them difficult to detect by commonly used techniques at concentrations useful for evaluating enzymatic metabolism. The methods under development last year proved too insensitive for metabolite analysis. We are having success with a fluorimetric method based on derivatization with 2-(4-carboxyphenyl)-6-N,N-diethylaminobenzofuran (Assaf et al., J. Chromatog. A, 869:243, 2000) using a carbodiimide-catalysed esterification that reacts at the alcoholic hydroxyl groups common to all the oxidative metabolites. The fluorescent reagent is not commercially available and had to be synthesized. Derivative structures are being confirmed by HPLC/Mass Spectroscopy. Sufficient material is now on hand to pursue metabolic work. Separation of the derivatized compounds is readily accomplished using a 150 x 4 mm C18, reverse phase HPLC column and gradient elution. Detection is by fluorescence with excitation at 387 nm and emission at 537 nm. Sensitivity is in the picogram/microliter range, a level that makes metabolite detection feasible. Thiodiglycol, thiodiglycol sulfoxide, thiodiglycol sulfone and 2-hydroxyethyl thioacetic acid are easily derivatized with this system. Thiodipropanol is a commercially available, easily derivatized internal standard.

Testosterone

An improved HPLC Method for the separation of testosterone and its metabolites was developed based on the method of Purdon and Lehman-McKeeman (1997). Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan). The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of pump (LC-10AT VP), a solvent proportioning valve (FCV-10AL VP), a degasser (DUG-14A), a Shimadzu auto injector (SIL-10AD VP), and a Shimadzu UV/VIS detector (SPD-10A VP). Data was collected via a Shimadzu system controller (SCL-10A VP) and analyzed using CLASS-VP 7.0 software. The mobile phase for pump A was 5% tetrahydrofuran, 95% water, and for pump B 100% methanol. A linear system was employed in the following manner: 0-6 min (30%B), 6-50 min (30-60% B), 50-55 min (60-90% B), 55-57 min (90% B), 57-58 min (90-30%B), and 58-60 min (30% B). The flow rate was 0.5 ml/min. Metabolites were separated by a C18 column (Luna 5 μ , 150 x 3 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 247 nm. A representative separation is shown in Figure 1.

Part 2. Tasks 1 through 4. These tasks cover, in toto, the kinetic analysis of substrates and isoforms active in their metabolism, the metabolism of test chemicals in phenotyped microsomes from individual humans and the effect of inhibitors of specific CYP isoforms and inhibitory potential of the test compounds. For ease of reporting and review they are presented compound by compound rather than function by function.

Chlorpyrifos:

Some of the studies of human chlorpyrifos metabolism reported in the last annual report have been published during the current reporting period (Tang et al., 2001, copy attached). These are the most complete in vitro human metabolism studies of an organophosphorus xenobiotic published to date and, as indicated, have important implications for possible interactions and for the definition of individuals and sub-populations at increased risk.

This latter point is further exemplified by the studies of variants of human CYP3A4, an isoform active in both the activation and detoxication of chlorpyrifos (Dai et al., 2001, copy attached). It is known that the expression of this isoform, often the most abundant CYP isoform in human liver can, nevertheless, vary as much as 40-fold between individuals. In the current studies several new polymorphic variants of CYP3A4 have been identified, sequenced, expressed and characterized. These variants differ from the wild type in their frequency in different populations and in their ability to metabolize chlorpyrifos.

In ongoing studies supported by another agency we have characterized the human metabolism of the carbamate insecticide, carbaryl, identifying the isoforms responsible for the production of the three major oxidative metabolites. Of relevance to this report, however, is the observation that chlorpyrifos is a potent inhibitor of carbaryl metabolism. Sample data sets are shown in the following tables (Tables 1 - 5). Note that inhibition at 0 time preincubation is due to a combination of irreversible and competitive inhibition taking place during the incubation period.

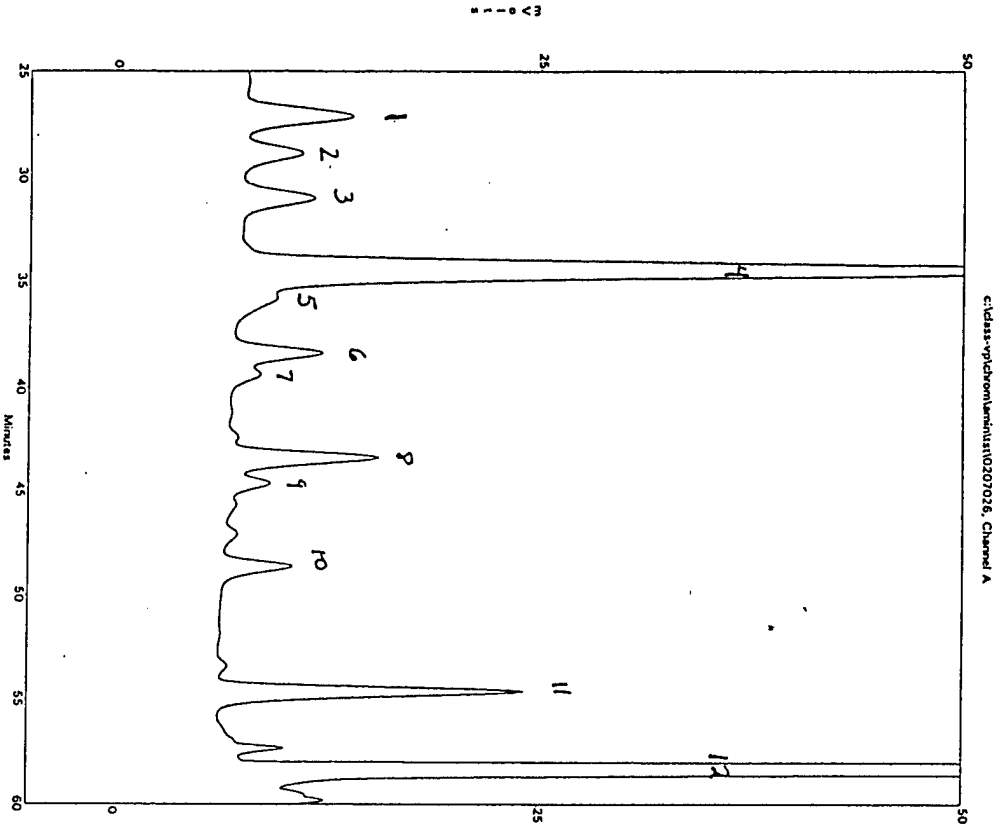


Fig 1. Representative chromatogram for testosterone metabolism in male CD-1 mice from Phenobarbital-induced microsomes*.

- 1) 7 β -Hydroxytestosterone
- 2) 6 α -Hydroxytestosterone
- 3) 15 β -Hydroxytestosterone
- 4) 6 β -Hydroxytestosterone
- 5) 15 α -Hydroxytestosterone
- 6) 7 α -Hydroxytestosterone
- 7) 16-ketotestosterone
- 8) 16 α -Hydroxytestosterone
- 9) 2 α -Hydroxytestosterone
- 10) 2 β -Hydroxytestosterone
- 11) Androstenedione
- 12) Testosterone

* Tentative identification of testosterone metabolites were made based on Purdon and Lehman-McKernan (1997) chromatograms. Further improvement in the separation of testosterone metabolites is underway.

Table 1. Inhibition of carbaryl metabolism by pre-incubation of pooled human liver microsomes with chlorpyrifos.

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	%inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
5	0	6	52
15	14	37	69
30	43	56	80

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and microsomal protein 1 mg in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
 2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.021 nmol/mg protein/min, 4-hydroxycarbaryl 0.45, carbaryl methylol 0.301.

Table 2. Inhibition of carbaryl metabolism by pre-incubation of human CYP1A1 with chlorpyrifos

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	%inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
0	87	89	33
30	96	95	58

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and 17.86 pmol CYP1A1 in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
 2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.002 nmol/mg protein/min, 4-hydroxycarbaryl 0.0039, carbaryl methylol 0.00012.

Table 3. Inhibition of carbaryl metabolism by pre-incubation of human CYP2B6 with chlorpyrifos.

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	% inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
5	100	100	100
15	100	100	100
30	100	100	100

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and 22.73 pmol CYP2B6 in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
 2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.0002 nmol/mg protein/min, 4-hydroxycarbaryl 0.0012, carbaryl methylol 0.0135.

Table 4. Inhibition of carbaryl metabolism by pre-incubation of human CYP3A4 with chlorpyrifos.

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	% inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
0	96	94	86
5	89	92	93
30	99	95	100

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and 25 pmol CYP3A4 in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
 2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.0014 nmol/mg protein/min, 4-hydroxycarbaryl 0.00444, carbaryl methylol 0.00059.

As discussed in the manuscript on DEET (Appendix 3), chlorpyrifos is also an inhibitor of the metabolism of DEET by human liver microsomes.

Chlorpyrifos studies were also supported, in part, by the North Carolina Environmental Trust Fund.

DEET:

A manuscript is now in press (Appendix 3) on the oxidative metabolism of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) by pooled human liver microsomes (HLM), rat liver microsomes (RLM), and mouse liver microsomes (MLM). Although these studies were largely described in the previous annual report and in Appendix 3, they are summarized, for the sake of continuity, at this point.

DEET is metabolized by cytochrome P450s (CYP) leading to the production of a ring methyl oxidation product, *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) and an *N*-deethylated product, *N*-ethyl-*m*-toluamide (ET). Both the affinities and intrinsic clearance of HLM for ring hydroxylation are greater than those for *N*-deethylation. Among 15 cDNA-expressed CYP enzymes examined, CYP1A2, 2B6, 2D6*1 (Val₃₇₄), and 2E1 metabolized DEET to the BALC metabolite while CYP3A4, 3A5, 2A6, and 2C19 produced the ET metabolite. CYP2B6 is the principal cytochrome P450 involved in the metabolism of DEET to its major BALC metabolite while CYP2C19 had the greatest activity for the formation of the ET metabolite. Use of phenotyped HLM demonstrated that individuals with high levels of CYP2B6, 3A4, 2C19, and 2A6 have the greatest potential to metabolize DEET. Mice treated with DEET demonstrated induced levels of the CYP2B family, increased hydroxylation, and a 2.4 fold increase in the metabolism of chlorpyrifos to chlorpyrifos-oxon, a potent anticholinesterase. Preincubation of human CYP2B6 with chlorpyrifos completely inhibited the metabolism of DEET. Preincubation

of human or rodent microsomes with chlorpyrifos, permethrin, and pyridostigmine bromide, alone or in combination, can lead to either stimulation or inhibition of DEET metabolism.

Permethrin:

Instrumentation used in the HPLC system for separation and quantitation of permethrin and metabolites was described in last year's progress report. Substantial revisions in the procedure were found to be necessary due to poor resolution of phenoxybenzoic acid. In an attempt to optimize the procedure, studies were conducted to optimize run time, wavelength and pH for resolution of each metabolite.

The optimal conditions for separation and resolution of permethrin and its metabolites involved the use of a gradient system. Two solvents (solvent A: 90% acetonitrile and 10% H₂O, solvent B: 100% H₂O adjusted to pH 1.7 with 85% phosphoric acid) were used for gradient elution (flow rate: 1ml/min). In cases where pH changes were necessary, the pH of solvent B was altered accordingly. The gradient system (linear increase) was initiated with 50% of solvent A and 50% of solvent B reaching 75% of solvent A at 6 min, and 100 % of solvent A at 7 min. One hundred percent of solvent A was maintained for 4 min and then reduced to 50% at 12 min, which was kept for 4 min to prepare the column for the initial conditions. The chromatographic analysis was conducted at ambient temperature.

The HPLC gradient system reliably resolved and detected the two permethrin isomers and three major metabolites. Using this method, phenoxybenzyl alcohol, phenoxybenzoic acid and phenoxybenzaldehyde were eluted at 3.7, 4.1 and 5.9 minutes respectively. The retention times for *trans* and *cis* permethrin were 10.2 and 10.5 minutes. To date, only one investigator has reported a HPLC method that analyzed all five compounds (Bast et al., 1997). In that study, the last peak eluted at 24.5 min, contrasting with this method in which a significant time savings was effected without loss of peak resolution.

To determine the optimal wavelength for detection of each standard, ten millimolar stock solutions of permethrin isomers and metabolites were prepared in acetonitrile (except for phenoxybenzyl alcohol which was prepared in methanol). Each was analyzed at four different wavelengths (210, 230, 254, and 270 nm). Each of these wavelengths have been used by previous investigators. Linear standard curve extrapolation for each compound was calculated using GraphPad Prism software for pc (GraphPad Software, Inc., San Diego, CA, USA).

Our analysis of each metabolite at several of these reported wavelengths showed 230 nm to be the most appropriate detection wavelength (Figure 2). The detection limit established for each compound was defined as the concentration at which the signal-to-noise ratio exceeded 3:1. The limit of quantitation was defined as the lowest concentration capable of generating a reproducible and predictable peak area. Although sensitivity to permethrin and its metabolites is greatest at 210 nm, lower detection and quantitation limits were obtained at 230 nm as shown in

Table 5, due to high levels of background solvent noise at 210 nm. Detection and quantitation limits significantly increased at detection wavelengths of 254 and 270 nm, making these wavelengths less appropriate for analyses requiring higher sensitivity. In the range between the detection limit and the highest concentration used (12.5 μM), each compound showed a strong and repeatable linear relationship between concentrations and peak areas at all wavelengths.

Since phenoxybenzoic acid is ionized at a neutral pH, the effect of mobile phase pH on the protonation of this and other metabolites was determined. Mobile phase pH was adjusted by increasing the hydrogen ion concentration in solvent B (pH levels of 1.7, 2.3, 3.2 and 8.0) with 85% phosphoric acid. Mobile phase hydrogen ion concentration clearly affected the resolution of ionizable metabolite (phenoxybenzoic acid). As shown in Figure 3, phenoxybenzoic acid did not form a distinguishable peak at the solvent B pH level of 8.0. As the protonation of phenoxybenzoic acid proceeded with increasing hydrogen ion concentration in the mobile phase, a separate peak of phenoxybenzoic acid gradually became distinguishable, forming a clearly identifiable peak at the lowest solvent B pH level of 1.7 among the four tested solvent B pH levels (Fig. 3).

Studies were also conducted to determine the extent to which permethrin is metabolized in human liver microsomes. In these studies, pooled liver microsomes obtained from Gentest (Woburn, MA, USA) were incubated with *trans*-permethrin. The reaction mixture of 500 μl in 0.1 M Tris buffer (pH 7.5) contained 0.5 mg of microsomal protein, 10 μl of *trans*-permethrin stock solution (10 mM in acetonitrile), and 35 μl of an NADPH regenerating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U glucose 6 phosphate dehydrogenase, final concentrations). All components except the liver microsomes were mixed and pre-incubated for 5 minutes at 37°C. One hundred minute incubations began with the addition of microsomes to the pre-warmed reaction mixture. After stopping the reaction with 500 μl of cold acetonitrile, the reaction mixtures were centrifuged at 20,000 g for 5 minutes. The supernatant was sampled and stored at 4°C until HPLC analysis.

Since preliminary HPLC analysis indicated no significant *cis*-permethrin metabolism, human metabolism studies were conducted with *trans*-permethrin. Figure 4 shows a typical metabolite profile of human liver microsomal metabolism of *trans*-permethrin. While phenoxybenzyl alcohol and phenoxybenzoic acid were detected, phenoxybenzaldehyde was barely detected in microsomal incubation. No permethrin metabolites were produced in the absence of human liver microsomes.

To determine the nature of the metabolic pathways leading to phenoxybenzyl alcohol and phenoxybenzoic acid, permethrin was incubated with a series of CYP isoforms expressed in baculovirus. None of the isoforms tested, including CYP1A1, 1A2, 2B6, 2C9, 2C18, 2C19, 2E1, 2D6 and 3A4 produced detectable metabolites. Although these metabolites were produced by the microsomal fraction and in the presence of an NADPH regenerating system, the lack of reactivity with these CYP isoforms indicated that pathways other than CYP pathways of metabolism should be considered.

Replacement of the NADPH regenerating system with NAD⁺ in incubations of *trans*-permethrin with pooled human liver microsomes produced more phenoxy-benzaldehyde and phenoxybenzoic acid than was observed previously. This finding indicated that phenoxybenzyl alcohol was sequentially oxidized by the NAD⁺ requiring alcohol and aldehyde dehydrogenases.

Subsequent incubations of phenoxybenzyl alcohol and phenoxybenzaldehyde with horse alcohol dehydrogenase (ADH) and yeast aldehyde dehydrogenase (ALDH) produced the expected products, phenoxybenzaldehyde and phenoxybenzoic acid, respectively. To further investigate the role of human ADH, four different human ADHS (α , β -I, β -II, δ) expressed in the *E. coli* strain (JM 105) using the pKK223-3 vector (Pharmacia), strain) were obtained from Dr. Alan Brimfield.

To utilize the alcohol dehydrogenases, it was necessary to produce the protein and purify it for expression studies. Purification methods were modified from those of Hurley et al. (1997). *E. coli* cells containing the appropriate plasmid DNA were grown in Terrific Broth and harvested according to previous methods (Stone et al., 1993) with some modifications. Four liters of the media contained 48 g of yeast extract, 48 g of peptone, 16 ml of glycerol and 400 ml of potassium phosphate buffer. The potassium phosphate buffer, consisting of 50 g of K₂HPO₄ and 9.2 g of KH₂PO₄ in 400 ml water, was prepared separately. The autoclaved solutions were mixed at room temperature. Ampicillin (200 mg) was added to the mixture (4 L). Following the inoculation with *E. coli*, cells were grown at 37°C until the absorbance reading reached 0.6-0.8 at 595 nm. Then, isopropylthiogalactoside (1.0 ml of 0.1 M stock per liter media) and ZnSO₄ (0.7 ml of 100 mM stock per liter media) were added to the media. Incubation temperature was reduced to the room temperature and cells were cultured an additional 12-24 hours prior to cell collection. Cells were collected using centrifugation (4,000g for 10 min). Collected cells were placed in ice and disrupted using a sonicator (550 sonic dismembrator, Fisher Scientific) in cell lysis buffer (pH 8.0, 10 mM Tris, 2 mM dithiothreitol (DTT), 0.1 mM ZnSO₄, 1 mM benzamidine). The cytosolic enzyme was obtained by collecting the supernatant after ultracentrifugation (100,000g for 35min at 4°C)(Beckman L7).

ADHs were purified using 3 chromatography columns: DEAE-cellulose (weak anion exchanger), SP-sepharose (strong cation exchanger) and Affi-Gel Blue columns (affinity gel for dehydrogenases and albumin). The supernatant was directly applied to DEAE-cellulose. Since the isoelectric point of beta ADHs is 8.6 (positively charged at pH 8.0), these ADHs flow through DEAE-cellulose column, which has a positive charge. Active fractions (measured by monitoring NADH formation in the presence of 33 mM ethanol at 340 nm) were collected and buffer-exchanged to SP-sepharose buffer (pH 8.0, 10 mM Hepes, 2 mM DTT, 0.1 mM ZnSO₄, 1 mM benzamidine) using ultrafiltration tubes (Centricon, Millipore). After loading the active fraction onto a SP-sepharose column, the column was thoroughly rinsed with column buffer prior to use of a NaCl gradient (0 - 250 mM) to remove the ADH proteins. Collected active fractions were buffer-exchanged to Affi-Gel Blue column buffer (pH 7.4, 10 mM Sodium Phosphate, 0.5 mM DTT, 0.1 mM ZnSO₄). ADH proteins bind to the Affi-Gel Blue column strongly and 0.8 M NaCl

solution prepared in the column buffer was used to elute bound proteins after thorough rinsing. Pooled active fractions were buffer-exchanged to Affi-Gel Blue column buffer and stored at -70°C with 50 % glycerol. Protein concentration was measured based on bicinchoninic acid reaction (Smith et al., 1985).

For alpha ADH, which has a lower isoelectric point than beta, the pH of the SP-sepharose buffer was lowered to 7.2. All other chromatography procedures were the same as mentioned for beta ADHs. For sigma ADH, the pH of the SP-sepharose buffer was 6.4 with 77 mM sodium phosphate, 1mM DTT, 0.1mM ZnSO_4 , 1mM Benzamidine). Sigma ADH from SP-sepharose column was eluted using 7 to 65mM sodium phosphate gradient. Affi-Gel Blue column buffer for the sigma ADH had the same composition with other isozymes with lower pH (6.4). Sigma ADH was eluted from Affi-Gel Blue column with column buffer to 100 mM Tris buffer (pH 8.8). The storage conditions for alpha and sigma isozymes were the same as for the beta ADHs. The purity of the ADH enzymes was evaluated by SDS-PAGE and staining with Coomassie Blue.

Purified ADH was assayed for phenoxybenzyl alcohol oxidation to phenoxybenzaldehyde to calculate K_m and V_{max} values based on Michaelis-Menten steady-state kinetics. A series of phenoxybenzyl alcohol concentrations were prepared in 100 mM Glycine buffer (pH 10.0) and mixed with NAD^+ (final concentration 2.5 mM). The reaction mixtures were pre-warmed to 25°C . The enzymatic reaction was started by addition of purified ADH to the reaction mixture. The reaction rate was calculated by measuring NADH formation by following the change in absorbance at 340 nm using a spectrophotometer (UV-2101PC, Shimadzu). Calculations of activity used the molar extinction coefficient of 6.22 mM/min/cm. Model fitting using GraphPad (GraphPad Software, San Diego CA) program was used to estimate K_m and V_{max} values.

The kinetic constants for phenoxybenzyl alcohol and ethanol are presented in Tables 6 and 7. The most significant difference between substrates is the much lower K_m values for phenoxybenzyl alcohol, as much as 300 times lower in case of beta-I. These data indicate that phenoxybenzyl alcohol is a significantly more preferred substrate for human ADH. This is in agreement with a previous study (Wagner et al., 1983 and Chen and Yoshida, 1991), which indicated that ADH isozymes show much higher affinity to substrates with large side chains.

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Table 5. Limits of detection and quantitation for permethrin isomers and their metabolites at different detection wavelengths based on the HPLC method described in the experimental section.

wavelength	compound	limit of detection (nM)	Limit of quantitation (nM)
210 nm	Phenoxybenzyl alcohol	120	120
	Phenoxybenzoic acid	24	49
	Phenoxybenzaldehyde	49	98
	Trans permethrin	24	49
	Cis permethrin	24	97
230 nm	Phenoxybenzyl alcohol	120	120
	Phenoxybenzoic acid	12	12
	Phenoxybenzaldehyde	12	24
	Trans permethrin	24	49
	Cis permethrin	24	49
254 nm	Phenoxybenzyl alcohol	490	980
	Phenoxybenzoic acid	98	390
	Phenoxybenzaldehyde	49	49
	Trans permethrin	195	390
	Cis permethrin	195	390
270 nm	Phenoxybenzyl alcohol	240	490
	Phenoxybenzoic acid	195	390
	Phenoxybenzaldehyde	195	195
	Trans permethrin	390	390
	Cis permethrin	390	390

Table 6. Kinetic constants of human alcohol dehydrogenase isozymes for phenoxybenzyl alcohol oxidation to phenoxybenzaldehyde

isozyme	Km (μ M)	SE Km	Vmax (nmoles/mg.min)	Vmax/Km
Alpha	33.66	3.43	2765	82145
Beta-I	3.74	0.86	389	104010
Beta-II	28.94	4.37	265.6	9177
Sigma	47.86	6.09	6452	134810

Table 7. Kinetic constants of human alcohol dehydrogenase isozymes for ethanol oxidation to acetaldehyde

isozyme	Km (mM)	SE Km	Vmax (nmoles/mg.min)	Vmax/Km
Alpha	1.57	0.18	3987	2539
Beta-I	1.12	0.16	540.6	483
Beta-II	4.75	0.507	657.4	138.4
Sigma	4.06	0.33	7752	1909

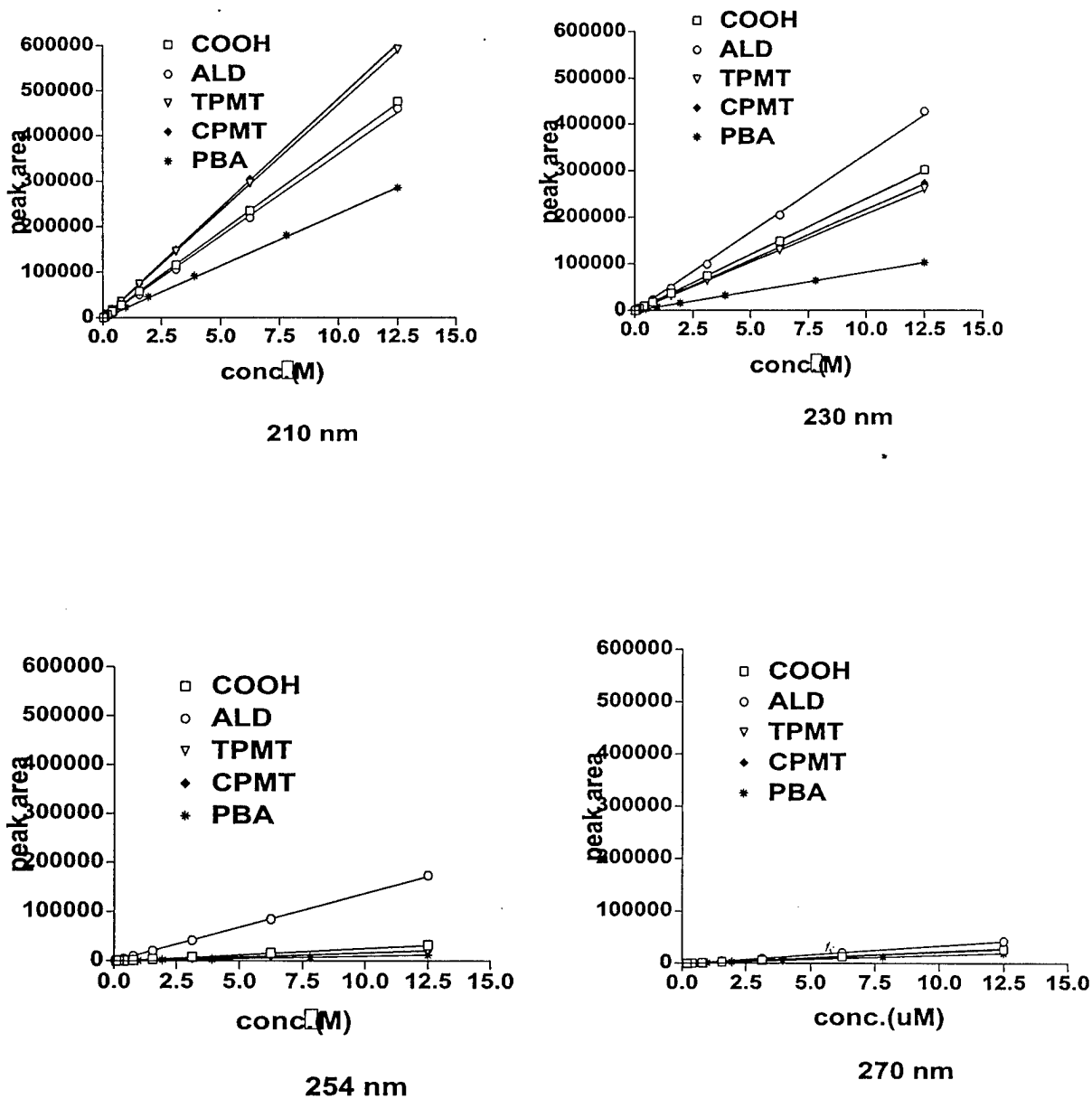


Figure 2. Standard calibration curves for permethrin isomers and their metabolites at four different wavelengths. COOH: phenoxybenzoic acid, ALD: phenoxybenzaldehyde, TPMT: trans-permethrin, CPMT: cis-permethrin, PBA: phenoxybenzyl alcohol.

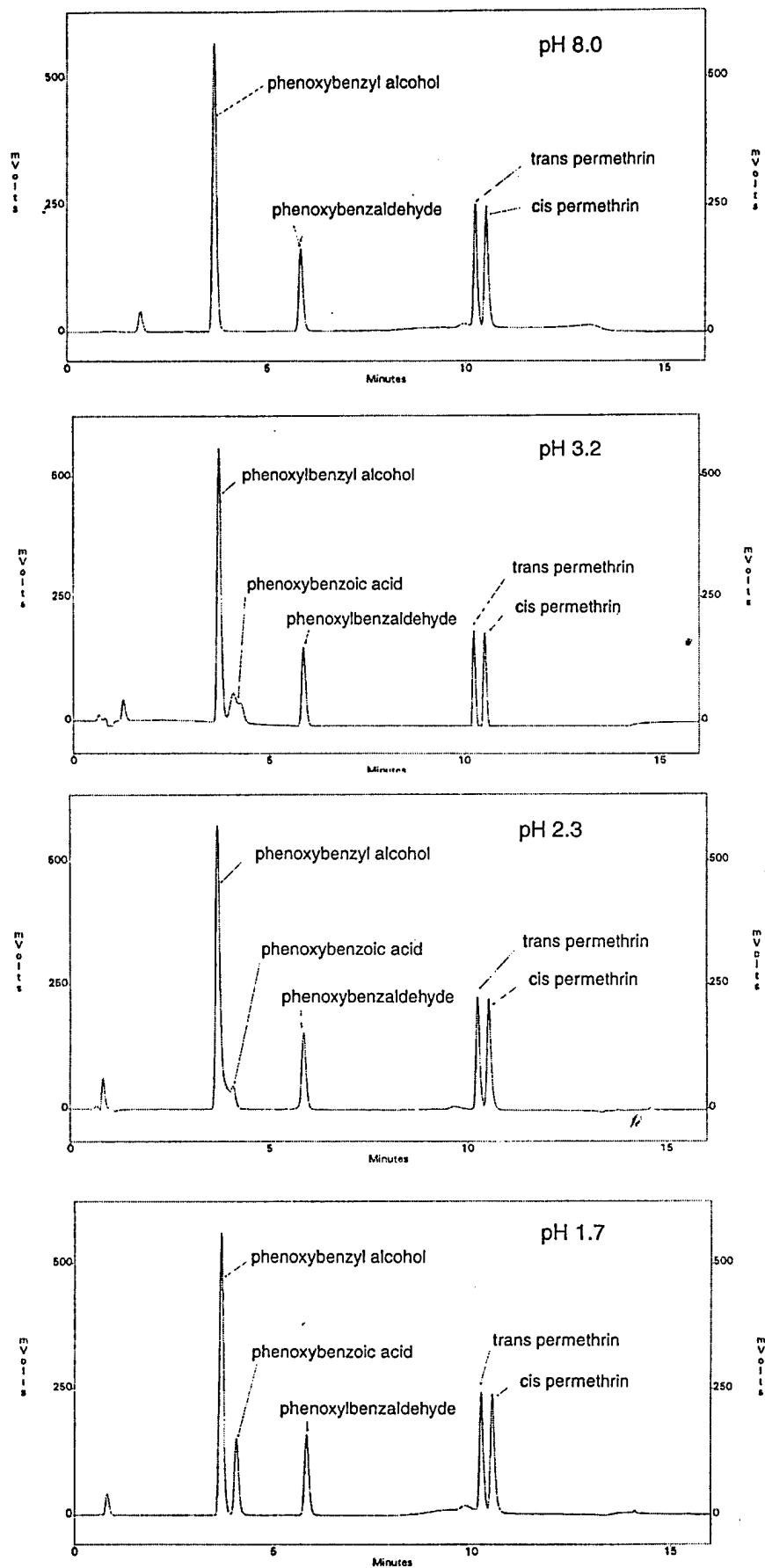


Figure 3. The effect of pH of the mobile phase solvent B on the resolution of permethrin isomers and their metabolites. The pH was adjusted with 85% phosphoric acid

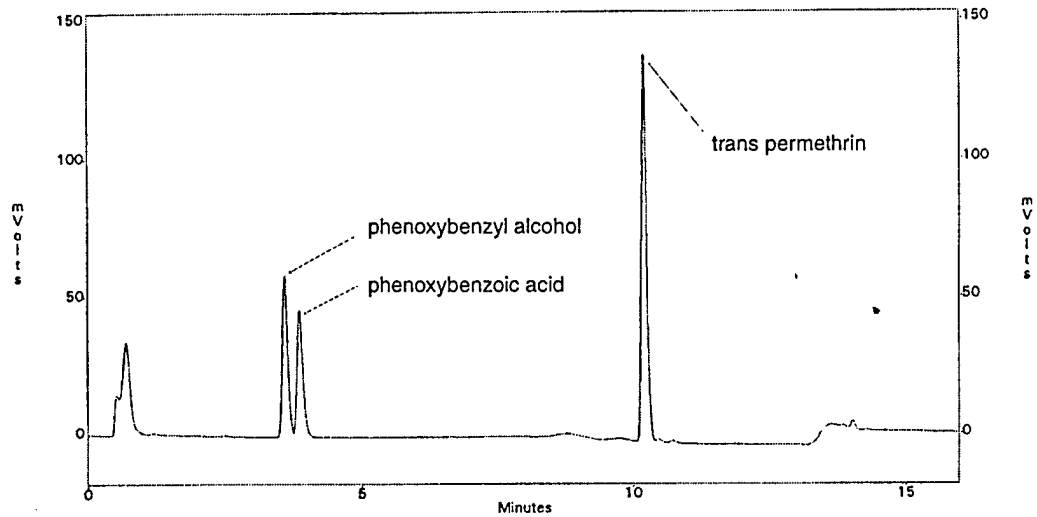


Figure 4. Metabolite profile of *trans*-permethrin incubation (1 hour and 40 minutes at 37C) with pooled liver microsomes.

K

Pyridostigmine bromide:

Pyridostigmine bromide metabolism activity assays were performed by incubation of 50 mM pyridostigmine bromide with pooled human liver microsomes, pooled human liver cytosol, pooled human liver S9, rat liver microsomes, rat liver cytosol, and rat serum for 60 min. The microsomal protein concentrations used in the assays were 1.0 mg/ml in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 5 mM MgCl₂ and 3 mM EDTA. Reactions were stopped by the addition of methanol followed by centrifugation at 15,000 rpm. The supernatant was analyzed for pyridostigmine bromide metabolites by HPLC.

Our data indicate that pyridostigmine bromide is not metabolized by pooled human liver microsomes, rat liver microsomes, pooled human liver cytosol, rat liver cytosol, pooled human liver S9, or rat serum. An earlier study by Leo et al., (1977) provided evidence that pyridostigmine bromide is not metabolized in humans and specifically that it is not a substrate for human CYPs 1A1, 2C9, 2E1, 2D6 and 3A4. This is confirmed and extended by our results.

Part 3. *Task 5. Continue induction experiments with mice*

Adult male CD-1 mice, 28-30 grams, were obtained from Charles River Laboratories and acclimated for 4 days. Doses for pyridostigmine bromide, trans- and cis-permethrin, and chlorpyrifos were selected such that the highest administered dose did not exceed doses known to produce physiological effects. The doses administered by intraperitoneal injection for each of three days as follows: pyridostigmine bromide (0.1, 0.5, and 1 mg/kg/day) in 100 µl water, trans-permethrin (1, 10, and 100 mg/kg/day) in 100 µl corn oil, cis-permethrin (1, 10, and 20 mg/kg/day) in 100 µl corn oil, chlorpyrifos (0.1, 1, and 10 mg/kg/day) in 100 µl corn oil. Doses approximating LD₁₀ values for phenobarbital (80 mg/kg/day) in 100 µl water or 3-methylcholanthrene (20 mg/kg/day) in 100 µl corn oil were also administered intraperitoneally, to separate groups of mice, daily for 3 days. Controls were given corn oil only or water.

Microsomes were prepared from livers of fed mice on the fourth day according to the method of Cook and Hodgson (1983). Total cytochrome P450 content was determined by the CO-difference spectrum method of Omura and Sato (1964). Protein concentration was determined using the BioRad protein assay with bovine serum albumin (BSA) as standard (Bradford, 1976).

The following substrates were used as indicators of the activities for the following isozymes: ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) for CYP1A1/2 (Burke and Mayer, 1974; Nerurkar et al., 1993), and pentoxyresorufin O-dealkylation (PROD) for CYP2B10 (Lubet et al., 1985). Assays were conducted as described by Pohl and Fouts (1980). Product formation for EROD, MROD and PROD activities were determined spectrofluorimetrically (RF-5301PC Personal Fluorescence spectrophotometer, Shimadzu, Kyoto, Japan) by comparison with a standard curve generated with resorufin.

Mice treated with PB, 3-MC, and varying concentration of pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos showed no significant differences in liver weight. PB treated mouse liver microsomes showed significantly higher P450 content than control or pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos treated mouse liver microsomes. No significant differences were observed in P450 content between control and pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos treated mouse liver microsomes. Mice treated with varying concentrations of pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos exhibited no significant levels of induction toward EROD, MROD, and PROD. Phenobarbital treatment as a positive control significantly induced PROD activity (12-fold). In a similar manner, treatment with 3-methylcholanthrene also resulted in significant induction of MROD (8-fold) and EROD (28-fold) as expected.

Sulfur Mustard and its Degradation Products:

There is no literature on the *in vitro* metabolism of these compounds. The first round of the induction of mice with thiodiglycol has been completed. This was a range finding experiment with 5 mice per dose level using ip injections of 5, 10, and 20% of an LD₅₀ dose daily for three days followed by liver microsome preparation on the fourth day and freezing of the microsomes at high protein concentration in phosphate buffer containing 0.25 M sucrose (Sabourin et al., Int J. Biochem., 16:713, 1984). The animals exhibited no signs of discomfort at the doses employed. There was evidence of liver hypertrophy at the highest dose. A mass induction will be carried out after evaluation of the outcome of the range finding using representative P450 substrates. The work will be repeated with sulfur mustard.

KEY RESEARCH ACCOMPLISHMENTS

- Analytical methods developed for the test compounds, chlorpyrifos, DEET, permethrin, pyridostigmine bromide sulfur mustard metabolites and testosterone are available and have been utilized in carrying out experiments proposed for year 2.
- The most complete description to date of the human metabolism of an organophosphorus toxicant has been carried out and has recently been published. Additional studies of human CYP3A4 polymorphisms involved in detoxication and activation of chlorpyrifos, have also been published.
- The first description of DEET metabolism by human enzymes has been accomplished and is now in press, including identification of the CYP isoforms involved in the production of the two principal metabolites. Induction of XMEs by DEET and inhibition of DEET metabolism is also reported.
- A detailed outline of the human metabolism of permethrin has been developed. Oxidative enzymes do not appear to be involved, metabolism appearing to be initiated by hydrolysis,

followed by metabolism of the resultant alcohol to the aldehyde by alcohol dehydrogenase and then to the acid by aldehyde dehydrogenase. This pathway has been confirmed by the use of purified human enzymes.

- Studies of induction in mice by DEET indicate that a number of monooxygenase reactions are induced, including the activation of chlorpyrifos to chlorpyrifos oxon.
- It has become clear that chlorpyrifos and, by implication, other organophosphorus compounds are potent inhibitors of the metabolism of other xenobiotics. Chlorpyrifos is shown to be a potent inhibitor of the human metabolism of DEET and the insecticide carbaryl.

REPORTABLE OUTCOMES

Publications, 2001 and in press - attached in full (see appendices).

Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Dai, D., Goldstein, J. A. and Hodgson, E. (2001) Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse and rat liver microsomes. *Drug Metabol. Disp.* 29:12-1-1204.

Dai, D., Tang J., Rose, R. L., Hodgson, E., Bienstock, R. J., Mohrenweiser, H. W. and Goldstein, J. A. (2001). Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. *J. Pharmacol. Exptl. Therap.* 299:825-831.

Usmani, K. A., Rose, R. L., Goldstein, J. A., Taylor, W. G., Brimfield, A. A. and Hodgson, E. (2002). In vitro human metabolism and interactions of the repellent, N,N-diethyl-m-toluamide (DEET). *Drug Metabol. Disp.* In press.

Presentations given and submitted:

Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Goldstein, J. A. and Hodgson, E. 2001. Metabolic activity of human cytochrome P450 isoforms toward chlorpyrifos. *The Toxicologist* 60: Abstract 242. SOT, San Francisco.

Usmani, K.A., R.L. Rose, J.A. Goldstein, and E. Hodgson. Metabolism of diethyl toluamide (DEET) by pooled human liver microsomes and human cytochrome P450 isoforms. Conference on Illnesses Among Gulf War Veterans: A Decade of Scientific Research. Alexandria, VA. Jan. 24-26, 2001.

J. Tang, Y. Cao, R.L. Rose, A.A. Brimfield, J.A. Goldstein, and E. Hodgson. Differential activities of chlorpyrifos metabolism in human liver microsomes and cytochrome P450 isoforms.

Conference on Illnesses Among Gulf War Veterans: A Decade of Scientific Research.
Alexandria, VA. Jan. 24-26, 2001.

CONCLUSIONS

HPLC analytical methods for sulfur mustard metabolites, pyridostigmine and testosterone have been brought on line and, together with those previously developed for chlorpyrifos, DEET and permethrin, have been used in several metabolic studies. The human cytochrome P450 isoforms metabolizing chlorpyrifos and DEET have been identified and include forms known to be inducible and forms known to be polymorphic. It has been established that pyridostigmine and permethrin are not significant substrates for monooxygenases. While pyridostigmine does not appear to be readily metabolized, permethrin has been shown to be metabolized, in humans, through a series of reactions involving hydrolytic enzymes, alcohol dehydrogenase and aldehyde dehydrogenase. Specific purified isoforms of human alcohol and aldehyde dehydrogenases have been shown to be active in these reactions. Chlorpyrifos is shown to be a potent inhibitor of DEET metabolism and of the metabolism of the insecticide, carbaryl. It is clear that chlorpyrifos and, by implication, other organophosphorus compounds, may be significant both in interactions between different deployment-related chemicals and in the determination of sub-populations and individuals at increased risk from anticholinergic chemicals.

These studies, the earlier studies on chlorpyrifos reported in the previous annual report and the successful completion of the remaining studies will permit more confident extrapolation of past and future animal studies to humans and permit identification of interactions not apparent from animal studies. They will also permit identification of human subpopulations at greater risk from specific xenobiotic toxicants and will produce specific analytic methodologies for assessment of future exposures.

It is becoming increasingly apparent that induction studies previously requiring the use of experimental animals may be accomplished directly with human materials. We continue to explore the possibility that newer techniques that maintain the capacity for induction in cultured human hepatocytes combined with microarray techniques for determination of gene expression and repression may substitute for the proposed animal studies. If this proves to be a viable approach, an appropriate proposal will be submitted.

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APPENDICES:

Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Dai, D., Goldstein, J. A. and Hodgson, E. (2001) Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse and rat liver microsomes. *Drug Metabol. Disp.* 29:12-1-1204.

Dai, D., Tang J., Rose, R. L., Hodgson, E., Bienstock, R. J., Mohrenweiser, H. W. and Goldstein, J. A. (2001). Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. *J. Pharmacol. Exptl. Therap.* 299:825-831.

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METABOLISM OF CHLORPYRIFOS BY HUMAN CYTOCHROME P450 ISOFORMS AND HUMAN, MOUSE, AND RAT LIVER MICROSOMES

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ABSTRACT:

One of the factors determining the toxicity of chlorpyrifos (CPS), an organophosphorus (OP) insecticide, is its biotransformation. CPS can be activated by cytochrome P450 (CYP) through a desulfuration reaction to form chlorpyrifos-oxon (CPO), a potent anticholinesterase. CPS can also be detoxified by CYP through a dearylation reaction. Using pooled human liver microsomes (HLM), a $K_{m,app}$ of 30.2 μ M and $V_{max,app}$ of 0.4 nmol/min/mg of protein was obtained for desulfuration, and a $K_{m,app}$ of 14.2 μ M and a $V_{max,app}$ of 0.7 nmol/min/mg of protein was obtained for dearylation. These activities are lower than those obtained from rat liver microsomes. Gender differences in humans were also observed with female HLM possessing greater activity than male HLM. Use of human CYP isoforms expressed in human lymphoblastoma cells demonstrated

that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 are involved in CPS metabolism. CYP2B6 has the highest desulfuration activity, whereas dearylation activity is highest for 2C19. CYP3A4 has high activity for both dearylation and desulfuration. The use of phenotyped individual HLM demonstrated that predictions of metabolic activation and/or detoxication could be made based on relative amounts of CYP2B6, 2C19, and 3A4 in the microsomes. Thus, individuals with high CYP2C19 but low 3A4 and 2B6 are more active in dearylation than in desulfuration. Similarly, individuals possessing high levels of CYP2B6 and 3A4 have the greatest potential to form the activation product. These differences between individuals suggest that differential sensitivities to CPS may exist in the human population.

Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] (CPS¹) is a widely used organophosphorus (OP) insecticide. It has numerous agricultural applications and, until recently, has been used for termite control in foundations and for the control of nuisance insects and disease vectors in homes and during military deployments. The extensive use of CPS inevitably results in human exposure and has the potential to cause toxic effects.

The *in vivo* toxicity of CPS is a result of its bioactivation by cytochrome P450 (CYP)-mediated monooxygenases to a more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO). This oxidation reaction, which proceeds through a possible phosphoxythiiran intermediate, can result in either a desulfuration reaction that generates the

oxon or a dearylation reaction that degrades the parent compound (Chambers, 1992) (Fig. 1).

Studies of parathion, a related organophosphate, have shown that parathion oxidation is catalyzed by human CYP1A2, 2B6, and 3A4 and that its oxidation is highly correlated to CYP3A4 activity in human liver microsomes (HLM) (Butler and Murray, 1997; Mutch et

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¹ Abbreviations used are: CPS, chlorpyrifos; CPO, chlorpyrifos-oxon; TCP, 3,5,6-trichloro-2-pyridinol; OP, organophosphorus; HLM, human liver microsomes; RLM, rat liver microsomes; MLM, mouse liver microsomes; CYP, cytochrome P450; HPLC, high-performance liquid chromatography.

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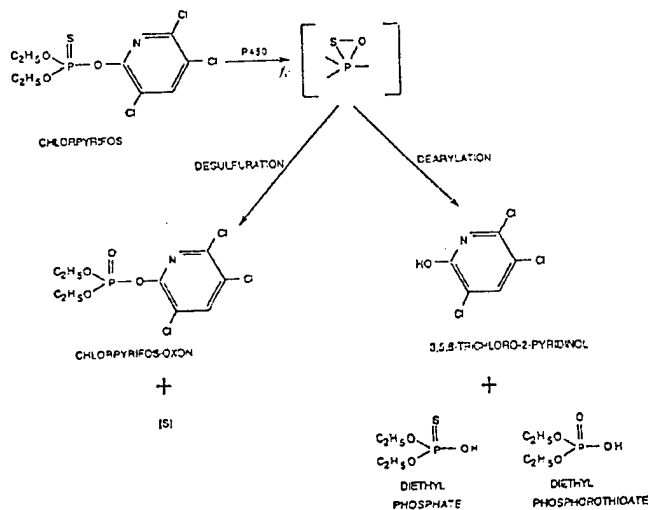


Fig. 1. Cytochrome P450-dependent metabolism of phosphorothioates illustrated with chlorpyrifos.

TABLE 1

Dearylation and desulfuration activities toward chlorpyrifos in HLM, RLM, or MLM

Activities are expressed as mean \pm S.E.M. ($n = 3$ determinations), means in RLM (pooled from three male rats) and MLM (pooled from three male mice) significantly different than HLM (pooled from 10 donors) are indicated by * $p < 0.05$ or ** $p < 0.01$.

	Desulfuration			Dearylation		
	$K_{m,app}$ μM	$V_{max,app}$ nmol/mg protein/min	$V_{max,app}/K_{m,app}$	$K_{m,app}$ μM	$V_{max,app}$ nmol/mg protein/min	$V_{max,app}/K_{m,app}$
HLM	30.2 \pm 1.7	0.4 \pm 0.1	0.01	14.2 \pm 2.2	0.7 \pm 0.1	0.05
RLM	6.1 \pm 1.1**	1.0 \pm 0.2*	0.17	4.8 \pm 1.8*	2.1 \pm 0.4**	0.43
MLM	24.4 \pm 2.4	0.7 \pm 0.1	0.03	14.9 \pm 3.0	2.7 \pm 0.1**	0.18

al., 1999). Other studies using specific chemical inhibitors for human CYP isoforms demonstrated that both CYP2D6 and CYP3A4 were active in the desulfuration of parathion, CPS, and diazinon (Sams et al., 2000).

The present study was designed to 1) determine oxidation activities toward CPS in human, mouse, and rat liver microsomes in the same assay system, 2) identify the human CYP isoforms and CYP polymorphic forms responsible for CPS oxidation, and 3) examine the differences in CPS oxidation activities among liver microsomes from selected individual humans.

Materials and Methods

Chemicals. CPS, CPO, and 3,5,6-trichloro-2-pyridinol (TCP) were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO).

Rodent Liver Microsome Preparation. Rat liver microsomes (RLM) and mouse liver microsomes (MLM) were prepared from adult male Long-Evans rats and adult male CD-1 mice (Charles River Laboratories, Raleigh, NC), respectively, according to the method of Cook and Hodgson (1983). Briefly, immediately after sacrificing the animals, the fresh livers were removed, weighed, minced, and then homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 1.15% potassium chloride. The homogenate was centrifuged at 10,000g for 15 min. The supernatant was filtered through glass wool and centrifuged at 100,000g for 1 h. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 0.25 M sucrose. All processes were performed at 0 to 4°C. The microsomal preparation was aliquoted and stored at -80°C until use. Protein concentration was determined using a BCA kit (Pierce, Rockford, IL).

Human Liver Microsomes and Human Cytochrome P450 Isoforms. Pooled HLM (pooled from 10 donors), individual HLM, and human lymphoblast-expressed CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1 (Arg₁₄₄, Ile₃₅₉), 2C9*2 (Cys₁₄₄), 2C19, 2D6*1 (2D6-Val), 2E1, 3A4, and 4A11 were purchased from GENTEST (Woburn, MA). Pooled male and pooled female HLM (pooled from 10 male donors and 10 female donors, respectively) were purchased from XenoTech, LLC (Kansas City, KS). Different mutant alleles of human CYP2C19 were expressed in *Escherichia coli*, according to the method of Luo et al. (1998). NADPH-CYP reductase was obtained from Oxford Biomedical Sciences (Oxford, MI).

In Vitro Chlorpyrifos Metabolism. Enzyme kinetic assays for microsomes were performed by incubation of serial concentrations of CPS (final concentration range, 2–100 μM) with microsomes in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 5 mM MgCl₂ and 3 mM EDTA for 5 min. The microsomal protein concentrations used in assays were 1.5 mg/ml for HLM, 0.5 mg/ml for RLM, and 1 mg/ml for MLM. After preincubation at 37°C for 3 min, reactions were started by the addition of an NADPH-generating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). The controls were identical except for the absence of an NADPH-generating system. Reactions were terminated by adding an equal volume of ice-cold methanol and vortexing. After 5 min of centrifugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for CPO and TCP concentrations by HPLC.

Metabolic activity assays for human lymphoblast-expressed CYP isoforms

were performed by incubation of CPS (final concentration, 100 μM) with CYP isoforms (final protein concentration, 0.9 mg/ml; final P450 contents, 23.4–180 pmol/ml) for 20 min in CYP-specific buffers recommended by the supplier (GENTEST). For CYP1A1, 1A2, 2D6, and 3A4, 100 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For CYP2B6, 2C8, 2C19, and 2E1, 50 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For CYP2C9*1, 2C9*2, and 4A11, the buffer was 100 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.5), whereas for CYP2A6, 50 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.4) was used.

The metabolic activity assays for *E. coli*-expressed human CYP2C19s were performed according to the method of Klose et al. (1998). Briefly, L- α -phosphatidylcholine (0.3 μg /pmol of P450), CYP reductase (4 pmol/pmol of P450), and CYP2C19 (24 pmol) were combined and preincubated at 37°C for 5 min. This mixture then was incubated with 100 μM CPS in 50 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) for 10 min. The reaction was initiated with NADPH-generating system as described previously. Assays of individual HLM (final protein concentration, 1.5 mg/ml) with CPS (final concentration, 100 μM) were described previously.

Analysis of Metabolites by HPLC. The HPLC system used in this study consisted of two Shimadzu (Kyoto, Japan) pumps (LC-10AT), a Shimadzu auto injector (SIL-10AD VP), and a Waters 486 tunable absorbance detector (Milford, MA). The mobile phase for pump A was 10% acetonitrile, 89% water, and 1% phosphoric acid, whereas that for pump B was 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min. Metabolites were separated by a C₁₈ column (Luna 5 μ , 150 \times 3 mm; Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm. Using this system, the retention times obtained for TCP, CPO, and CPS were 8.5, 12, and 17 min, respectively. The limits of detection for TCP and CPO were 0.03 and 0.04 μM , respectively, at an injection volume of 15 μ l. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. $K_{m,app}$ and $V_{max,app}$ were obtained using a Hanes-Woolf plot (Segel, 1975).

Statistics. Significant differences between data sets were determined by one-way analysis of variance, and multiple comparisons were performed with the Tukey-Kramer method using an SAS program (SAS, 1989).

Results

The protein concentrations and incubation times used in the assays were within linear ranges determined in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

HLM displayed lower affinity (i.e., higher $K_{m,app}$) and lower reaction velocity toward CPS for both desulfuration and dearylation than RLM (Table 1). Compared with HLM, MLM exhibited similar affinities but a higher reaction velocity toward CPS (Table 1). Both RLM and MLM have higher values of clearance terms ($V_{max,app}/K_{m,app}$) than HLM. Pooled female HLM showed significantly higher metabolic activity toward CPS than pooled male HLM (Table 2).

A screen of several human CYP isoforms demonstrated that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 were involved in CPS metabolism (Table 3), whereas no oxidation activity toward CPS was detected using CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Desulfuration and dearylation activities were greatest for CYP2B6

TABLE 2

Metabolic activities toward chlorpyrifos in pooled male and female HLM

Activities are expressed as mean ± S.E.M. (n = 3 determinations), means in female HLM (pooled from 10 donors) significantly different than male HLM (pooled from 10 donors) are indicated by *p < 0.05 or **p < 0.01. Protein concentration and cytochrome P450 content in microsomes are provided by the supplier (XenoTech, LLC).

	Desulfuration		Dearylation	
	nmol/mg protein/min	nmol/nmol P450/min	nmol/mg protein/min	nmol/nmol P450/min
Male	0.24 ± 0.01	0.54 ± 0.01	0.47 ± 0.01	1.05 ± 0.03
Female	0.56 ± 0.02**	0.89 ± 0.02**	0.78 ± 0.02**	1.23 ± 0.04*

TABLE 3

Metabolic activities toward chlorpyrifos in human lymphoblast-expressed cytochrome P450 isoforms

Activities are expressed as nanomoles of product per nanomoles of P450 per minute ± S.E.M. (n = 3 determinations).

	Desulfuration*	Dearylation*	Desulfuration/Dearylation
1A2	0.39 ± 0.06 ^a	0.23 ± 0.05 ^a	1.70
2B6	1.59 ± 0.12 ^b	0.47 ± 0.03 ^a	3.38
2C9	0.32 ± 0.06 ^a	0.90 ± 0.10 ^{ab}	0.36
2C19	0.30 ± 0.08 ^a	2.09 ± 0.42 ^b	0.14
3A4	0.52 ± 0.13 ^a	0.79 ± 0.24 ^{ab}	0.66

* Means in the same column followed by the same letter are not significantly different, p < 0.01.

and CYP2C19, respectively. Marked decreases in metabolic activity toward CPS were observed with different polymorphic alleles of CYP2C19 (Table 4).

To determine the variation range of CPS metabolism between individuals, we examined CPS metabolism from five individuals representing contrasting activities of some important CYP isoforms (Table 5). Individuals with high levels of CYP2B6 and 3A4 (HG042 and 112) had high-desulfuration activity; individuals with low levels of CYP2B6 and 3A4 (HG006, 023 and 043) had low-desulfuration activity. The dearylation pathway was more predominant in the individual (HG043) with high-CYP2C19 but low-3A4 levels. No particular increase in either metabolite was observed in the individual (HG023) with high levels of CYP2D6.

Discussion

The metabolic intrinsic clearance rates (V_{max}/K_m) indicate that liver microsomes from all three species more readily produce a detoxication product (i.e., TCP) than an activation product (i.e., CPO). These observations are similar to previous reports on rodents (Sultatos and Murphy, 1983; Ma and Chambers, 1995). The clearance rates also demonstrate that pooled HLM are less active than RLM and MLM in both desulfuration and dearylation, suggesting that less TCP and CPO are generated in the human liver than in the rodent liver immediately after exposure to CPS.

Consistent with data on other substrates provided by the supplier (XenoTech, LLC) regarding gender differences in CYP activity, pooled female HLM showed higher activities in both desulfuration and dearylation of CPS than pooled male HLM. Note that this gender difference was demonstrated using only one pool of 10 males and 10 females, respectively. It is not known whether this difference would also be true in a larger population. These data contrast with CYP activities in rats because males are more active in CPS desulfuration than females (Chambers and Chambers, 1989; Sultatos, 1991).

Our results show that human lymphoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 are responsible for both dearylation and desulfuration of CPS, whereas CYP1A1, 2A6, 2C8, 2C9*2, 2D6*1,

TABLE 4

Metabolic activities toward chlorpyrifos in CYP2C19 expressed in E. Coli

Activities are expressed as nanomoles of product per nanomoles of P450 per minute ± S.E.M. (n = 3 determinations). Means in the same column followed by the same letter are not significantly different, p < 0.01.

	Desulfuration	Dearylation
	nmol product/nmol P450	
2C19*1B	0.68 ± 0.03	6.11 ± 0.28 ^a
2C19*8	not detected	1.66 ± 0.07 ^b
2C19*6	not detected	0.69 ± 0.05 ^c
2C19*5	not detected	0.68 ± 0.06 ^c

TABLE 5

Metabolic activities toward chlorpyrifos in individual human liver microsomes

Activities are expressed as mean ± S.E.M. (n = 3-4 determinations). Individual human liver microsomes (protein concentration, 20 mg/ml). CYP2B6, 2C19, 2D6, and 3A4 activities (pmol/mg of protein/min), represented by (S)-mephenytoin N-demethylase, (S)-mephenytoin 4'-hydroxylase, bufuralol 1'-hydroxylase, and testosterone 6β-hydroxylase catalytic activities, 2990 and 2990 for HG006 (16-year-old male), 12.2, 78.1, respectively, are 3.1, 36, not detectable and 2990 for HG042 (48-year-old male), 160 and 4050 for HG023 (25-year-old male), 140, 3.5, 110 and 14530 for HG043 (23-year-old female), 59.1, 260.0, 23 and 17519 for HG112 (2-year-old female) (data were provided by GENTEST).

	Desulfuration*	Dearylation*
	nmol/mg protein/min	
HG006	0.09 ± 0.01 ^a	0.35 ± 0.03 ^a
HG023	0.16 ± 0.01 ^a	0.31 ± 0.04 ^a
HG042	0.74 ± 0.10 ^b	0.67 ± 0.07 ^{ab}
HG043	0.08 ± 0.01 ^a	0.61 ± 0.04 ^{ab}
HG112	0.67 ± 0.08 ^b	0.91 ± 0.10 ^b

* Means in the same column followed by the same letter are not significantly different, p < 0.01.

2E1, and 4A11 did not display detectable activities toward CPS oxidation. These results are similar to those of a parathion metabolism study (Butler and Murray, 1997), where CYP1A2, 2B6, and 3A4 were shown to have high-desulfuration activities toward parathion. CYP2B6 more readily generates the oxon, similar to phenobarbital-induced CYP2B isoforms in rodents (Fabrizi et al., 1999; Levi et al., 1988). CYP2C19 exhibits the greatest dearylation activity and relatively low-desulfuration activity. Genetic polymorphisms have been identified in CYP2C19 (Demorais et al., 1994), and differential metabolic activities toward CPS by different variants of CYP2C19 were observed in this study. Dearylation by the polymorphic CYP2C19 alleles was significantly less than that of the wild-type forms, which could influence the in vivo toxicity of CPS in individuals possessing these alleles.

CYP3A4 is also a highly active, although not the most active, isoform in CPS metabolism. The fact that CYP3A4 is the most abundant CYP isoform in human liver (Shimada et al., 1994) suggests that this isoform plays a significant in vivo role in both desulfuration and dearylation. CYP2C9*1 (Arg₁₄₄) showed some activities in both desulfuration and dearylation of CPS, whereas CYP2C9*2 (Cys₁₄₄), a single amino acid difference, showed no detectable activity toward CPS. CYP2C9*1 and 1A2, although not the most active isoforms for either desulfuration or dearylation, may play a role in vivo. A previous study using specific chemical inhibitors and human lymphoblastoid cell-expressed CYP2D6 suggested that CYP2D6 plays a significant role in desulfuration of parathion, CPS, and diazinon (Sams et al., 2000). Our work with CPS did not reveal any metabolites using human lymphoblast-expressed CYP2D6, nor did HLM with high-CYP2D6 activity (HG023) produce a significant amount of the oxon metabolite.

Potential differences in the human population with respect to CPS metabolism were further examined using different individual HLM.

Individuals with varying levels of CYP2B6, 2C19, 2D6, and 3A4 were selected to represent contrasting levels of predicted metabolic activity. Thus, individuals (HG042 and HG112) possessing high levels of CYP2B6 and CYP3A4 would be expected to possess greater ability to form the desulfuration product than those (HG006, HG023, and HG043) with lower levels of these isoforms, as observed (Table 5). Similarly, individuals with greater levels of CYP2C19 or CYP3A4, such as HG042, HG043, and HG112, would also be expected to produce more of the dearylation product than those with significantly lower levels of these isoforms (HG006 and HG023). Because individuals with contrasting levels of CYP2B6 and 3A4 were not available, we were unable to differentiate the extent of their contribution to desulfuration separately. However, based on its content in human liver, CYP3A4 should contribute significantly to both desulfuration and dearylation, as has been previously observed (Butler and Murray, 1997; Mutch et al., 1999; Sams et al., 2000).

Using our selection of five individuals, the variations between individual HLM in desulfuration of CPS were around 8-fold and that for dearylation was 3-fold and would presumably be greater if more samples were examined. For parathion, the difference between individual HLM in dearylation and desulfuration is as great as 10- and 16-fold, respectively (Butler and Murray, 1997; Mutch et al., 1999). Considerations of metabolic differences between individuals should also consider the contributions of esterases, which are also major factors determining the in vivo toxicities of OP compounds (Maxwell et al., 1987; Chambers et al., 1990; Costa et al., 1990).

In conclusion, HLM use the same pathways as RLM and MLM to metabolize CPS, although activities were generally lower in humans than in rodents. From pools of 10 individuals, female HLM displayed a higher activity in CPS metabolism than male HLM. Human lymphoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 showed oxidation activities toward CPS, whereas no activities were detected for CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Although CYP2C19 and 2B6 displayed the greatest dearylation and desulfuration activities, respectively, 3A4 was highly active in both reactions. Activities of CYP2B6, 2C19, and 3A4 greatly affect CPS metabolism in HLM.

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Identification of Variants of CYP3A4 and Characterization of Their Abilities to Metabolize Testosterone and Chlorpyrifos

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ABSTRACT

CYP3A4 is the most abundant isoform of cytochrome P450 (CYP) in adult human liver. It metabolizes numerous clinically, physiologically, and toxicologically important compounds. The expression of CYP3A4 varies 40-fold in individual human livers, and metabolism of CYP3A4 substrates varies at least 10-fold in vivo. Single nucleotide polymorphisms (SNPs) in CYP3A4 were identified by direct sequencing of genomic DNA in 72 individuals from three different ethnic groups, including Caucasians, Blacks (African-Americans and African pygmies), and Asians. A total of 28 SNPs were identified, including five which produced coding changes M445T (CYP3A4*3), R162Q (CYP3A4*15), F189S (CYP3A4*17), L293P (CYP3A4*18), and P467S (CYP3A4*19). The latter four represent new allelic variants. Racial variability was observed for the frequency of individual SNPs. CYP3A R162Q was identified only in Black populations

with an allelic frequency of 4%. CYP3A4 F189S and CYP3A4 M445T were identified in Caucasians with allelic frequencies 2% and 4%, respectively. L293P and P467S were only observed in Asians at allelic frequencies of 2%. The cDNAs for the F189S, L293P, M445T, and P467S mutant alleles were constructed by site-directed mutagenesis and expressed in an *Escherichia coli* expression system. Testosterone and the insecticide chlorpyrifos were used to assess the catalytic activities of the most common CYP3A4 allele (CYP3A4*1) and its allelic variants. CYP3A4 F189S exhibited lower turnover numbers for testosterone and chlorpyrifos, while CYP3A4 L293P had higher turnover numbers for both substrates. The turnover numbers of the CYP3A4 M445T and P467S alleles to metabolize these compounds were not significantly different from those of wild-type CYP3A4.

The CYP3A genes encode the most abundant CYP enzymes in humans including CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (de Wildt et al., 1999; Gellner et al., 2001). Hepatic CYP3A4 has been estimated to metabolize ~50% of currently used drugs as well as a number of steroids, environmental chemicals, and carcinogens (Aoyama et al., 1989; Shimada et al., 1994; Thummel et al., 1996; Rebbeck et al., 1998; Guengerich, 1999). CYP3A4 is considered to be the predominant form in adult human liver. CYP3A5, a polymorphic form, is present to a variable extent in adult livers. CYP3A5 is believed to be present in the livers of approximately 20% of Caucasians, but a recent study suggests that CYP3A5 is

expressed and may predominate in more than 50% of African-Americans (Lown et al., 1994; de Wildt et al., 1999; Wandel et al., 2000; Kuehl et al., 2001). Both CYP3A4 and CYP3A5 are distributed in multiple tissues including not only liver, but also intestine and kidney (Thummel and Wilkinson, 1998; Guengerich, 1999). CYP3A7 is an isoform found in intestine, reproductive organs, and infant liver but is also present in some adult livers (Kitada et al., 1985; Schuetz et al., 1994). Recently, a new CYP3A member (CYP3A43) has been identified. CYP3A43 mRNA is found predominantly in adult prostate and is also present in multiple tissues, including liver, where it is inducible by rifampicin (Gellner et al., 2001). However, Westlind et al. (2001) using heterologous expression systems including yeast, COS-1 cells, mouse hepatic H2.35 cells, and human embryonic kidney 293 cells suggested that CYP3A43 was a non-functional isoform.

CYP3A levels fluctuate in the liver throughout the life span of an individual (Shimada et al., 1994; Oesterheld,

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ABBREVIATIONS: CYP, cytochrome P450; SNP, single nucleotide polymorphism; OPs, organophosphorus; TLC, thin layer chromatography; TCP, trichloropyridinol; CPO, chlorpyrifos-oxon; PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography; NADPH, β -nicotinamide adenine dinucleotide phosphate, reduced form; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

1998). Up to 40-fold interindividual variations in expression levels of CYP3A4 have been observed in human liver. There is an approximately 10-fold variation in metabolism of CYP3A4 substrates in vivo including the antibiotics rifampicin and ketoconazole, the calcium blocker nifedipine, and the immunosuppressant cyclosporine (Thummel and Wilkinson, 1998; Guengerich, 1999). This variation can affect drug efficacy and toxicity. CYP3A4 is inducible by drugs such as rifampicin (Kolars et al., 1992). The variable expression of CYP3A4 is at least partially due to multiple factors, including induction by drugs, endogenous compounds, and environmental chemicals, but also includes genetic factors. Recent evidence suggests that the coding region of CYP3A4 is also genetically variable (Sata et al., 2000; Eiselt et al., 2001).

CYP3A4 has also been shown to be important in the metabolism of organophosphate pesticides (OPs), such as chlorpyrifos (Tang et al., 2001) and parathion (Butler and Murray, 1997; Eaton, 2000). Chlorpyrifos is a widely used broad-spectrum OP insecticide that elicits toxicity through inhibition of acetylcholinesterase (Chambers, 1992). OPs inhibit acetylcholinesterase and exert their toxicity by causing the accumulation of the neurotransmitter acetylcholine at nerve synapses and neuromuscular junctions. These OPs are used as the phosphorothioate ($P = S$), which is a very weak inhibitor of acetylcholinesterase. However, OPs are converted in vivo from ($P = S$) to an active phosphate ester or oxon ($P = O$), which is a potent acetylcholinesterase inhibitor (Chambers, 1992) by CYP enzymes.

Genetic variations of CYP3A4 have recently been reported. A mutation in the 5'-upstream region termed *CYP3A4*1B* (A290G) was observed in 52% of African-Americans and 9.6% of Caucasians, but has not been identified in Asians (Ball et al., 1999; Rebbeck, 2000; Sata et al., 2000; Gellner et al., 2001). It was suggested to be associated with advanced stage prostate cancer in men (Rebbeck et al., 1998), yet has protective effects for secondary cancer caused by chemotherapeutic drugs for leukemia metabolized by CYP3A4, such as epipodophyllotoxins (Felix et al., 1998). However, this polymorphism does not appear to affect constitutive levels of CYP3A4 (Wandel et al., 2000). Gonzales and coworkers (Sata et al., 2000) have described two coding SNPs including *CYP3A4*2* (S222P) found only in Finnish Caucasians with an allelic frequency of 2.7%, and a single case of *CYP3A4*3* (M445T) in a Chinese population of 178 individuals. Baculovirus expressed CYP3A4*2 protein exhibited an increase in the K_m for nifedipine but not for testosterone compared with CYP3A4*1. There has been limited information about the effects of a new M445T allele on metabolism (Sata et al., 2000). *CYP3A4*4* (I118V), *CYP3A4*5* (P218R), and *CYP3A4*6* (a stop codon at 285) were reported in a Chinese population with allelic frequencies of 1.4%, 0.98%, and 0.5%, and all of these variant alleles were associated with lower ratios of 6 β -hydroxycortisol to free cortisol in an in vivo study (Hsieh et al., 2001). A very recent study has identified seven new polymorphisms in European Caucasians (Eiselt et al., 2001).

To identify CYP3A4 polymorphisms, we screened for single nucleotide polymorphisms among 72 individuals from three different racial groups including ethnically diverse Caucasians, Blacks (African-Americans and African pygmies) and ethnically diverse Asians. We identified four new coding polymorphisms in CYP3A4. A bacterial cDNA expression system was used. Catalytic activities of wild-type CYP3A4

and allelic variants were compared using testosterone and the insecticide chlorpyrifos as prototypic 3A4 substrates.

Materials and Methods

Testosterone, β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), isopropyl β -D-thiogalactopyranoside, δ -aminolevulinic acid, phenylmethylsulfonyl fluoride, phosphatidylcholine, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). 14 C-Testosterone was purchased from Invitrogen (Boston, MA). Chlorpyrifos, chlorpyrifos-oxon (CPO), and 3,5,6-trichloro-2-pyridinol (TCP) were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Human NADPH reductase was obtained from Oxford Biomedical Research (Oxford, MI). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). Taq polymerase was purchased from QIAGEN (Valencia, CA). Anti-CYP3A4 antibody was obtained from GENTEST (Woburn, MA).

Direct Sequencing. Genomic DNA was obtained from 72 different human lymphoblastoid cell lines (Cornell Institute, Camden, NJ). The individuals contain the following varied racial and ethnic ancestries: 24 Africans (16 African-Americans and 8 African pygmies), 24 Asians (5 native Taiwanese, 5 mainland Chinese, 4 Melanesian, 4 Indo-Pakistani, 3 Cambodian, and 3 Japanese), and 24 Caucasians (9 from Utah, 5 Druze [Lebanon], 5 eastern European, and 5 from Moscow).

Variant Identification. The exons plus splice junctions and the 5' and 3' regions of CYP3A4 were sequenced as previously described by Shen et al. (1998). Briefly, PCR primers were located so that amplification of the genomic sequence is initiated approximately 50 nucleotides from each intron-exon boundary. This is sufficient distance for high quality sequence data to be obtained before reaching the intron/exon splice site. Appended to the 5'-end of each of the PCR primers were sequences containing the primer binding sites for the forward or reverse energy transfer DNA sequencing primers (Amersham Pharmacia Biotech, Cleveland, OH). The amplification products are directly sequenced according to the manufacturer's instructions using the DYEnamic Direct cycle sequencing kit with the DYEnamic energy transfer primers (Amersham Pharmacia Biotech). The denatured products are loaded onto ABI Prism 377 stretch DNA sequencers (Foster City, CA). "PolyPhred" (version 2.1), a software package that utilizes the output from Phred, Phrap, and Consed, was used to identify single nucleotide substitutions in heterozygous individuals (Nickerson et al., 1997; Rieder et al., 1998). A nucleotide sequence analysis program (<http://genomic.sanger.ac.uk/gf/gfl.html>) was used to predict possible new splice sites introduced by any new mutations.

Modification of CYP3A4 cDNA. CYP3A4 wild-type cDNA in the vector pUC19 was generously supplied by Frank Gonzales (National Cancer Institute, National Institute of Health). N-Terminal modification of CYP3A4 cDNA included removal of the initial 10 amino acids and conversion of the first eight amino acids of CYP3A4 into those of bovine 17 α -hydroxylase (MALLLAVF). This was accomplished by PCR using sense primer: 5'-TTAGGAGGTCATATGCTCTGTTATTAGCAGTTTTTCTGGTGCTCCTCTAT-3', which introduced a unique restriction site for *NdeI*. The antisense primer (5'-AGCAGAAGTCTCTAGAAAAATTCAGGCTCCACTTACGGTGC-3') was used to introduce an *EcoRI* site. *NdeI* and *EcoRI* sites are unique for the expression vector pCW. Amplification of CYP3A4 ORF was accomplished by PCR with Pfu polymerase using primers described above. PCR products containing an open reading frame of CYP3A4 were digested by *NdeI* and *EcoRI* and then were subcloned into pCW. Fidelity of PCR was verified by complete sequencing of CYP3A4. Sequentially, the plasmids were transformed into *E. coli* XL1 Blue cells.

Site-Directed Mutagenesis. Five mutations containing R162Q, F189S, L293P, M445T, and P467S were made using site-directed mutagenesis. A Chameleon double-stranded site-directed mutagen-

esis kit from Stratagene (La Jolla, CA) was used to introduce single nucleotide changes (indicated in lower case and boldface): primer 5'-GGTGAGAAATCTGAGGC**Ca**GGAAGCAGAGACAGG-3', was used to produce the substitution R162Q. The primer 5'-GTGATCACTAGCACATCA**Tc**TGGAGTGAACATCGACTC-3' was used to substitute individual nucleotide changes coding for the F189S substitution in exon 7. Primer 5'-CAAAGCTCTG**TCC**GATC**Cc**GGAGCTCGTG-GCCCAATC-3' introduced the substitution L293P in exon 10. Primer 5'-CTGCATTGG**Ca**cGAGGTTTGTCTC-3' introduced M445T in exon 12. Primer 5'-CAGA**ACTT**CTCCTTCAA**At**CTTGTA-AAGAAACACAGATCCC-3' introduces P467S in exon 12. The entire coding region, including the mutated sites, was verified by sequencing with an ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences, Foster City, CA). The entire cDNA was then excised and subcloned into a new pCW plasmid to avoid any accidental mutations in the plasmid caused by the mutagenesis procedure and expressed in *E. coli* XL1 Blue.

Expression and Partial Purification of CYP3A4s. Wild-type and variant CYP3A4 alleles were expressed in *E. coli* XL1 Blue and the allelic proteins were purified as previously described (Dai et al., 2001). Cytochrome P450 content was monitored by the reduced CO spectrum using a DW-2000 Spectrophotometer. Protein concentration was determined by the method of Lowry (Dawson and Heatlie, 1984).

Western Blot Analysis. SDS-polyacrylamide gel electrophoresis was used to separate the recombinant proteins, followed by transferring the proteins onto nitrocellulose membranes. Nonspecific binding was blocked by 10% nonfat milk for 1 h. The membranes were incubated with anti-CYP3A4 primary antibody for 1 h at room temperature. An enhanced chemiluminescent kit (Pierce, Rockford, IL) was used for immunodetection.

Testosterone Metabolism. Metabolism of testosterone by the recombinant wild-type and mutant CYP3A4 alleles was characterized. The purified recombinant CYP3A4 proteins (10 pmol) were reconstituted in with 0.4% CHAPS, 1 μ g of dioleoylphosphatidylcholine, and 40 pmol of human NADPH reductase (Oxford Biomedical Research, Oxford MI) and 20 pmol of cytochrome *b₅* in 1 \times HEPES buffer, pH 7.6 (50 mM HEPES, 15 mM MgCl₂, and 0.1 mM EDTA) a 10- μ l volume. The reconstitution mixture was preincubated at 37°C for 5 min and then diluted to a final volume of 100 μ l with 1 \times HEPES containing 10 μ g of dioleoylphosphatidylcholine. The optimal conditions for this substrate were generously provided by Drs. Halpert and He at the University of Texas Medical School in Galveston Texas. The reaction mixture was preincubated at 37°C for 5 min, and the reaction initiated by addition of 10 μ l of 10 mM NADPH and terminated with 50 μ l of tetrahydrofuran. All incubations were performed in triplicate. Samples were analyzed by thin layer chromatography (TLC) using a solvent system of dichloromethane/acetone (4:1, v/v). Finally, the TLC plate was exposed to radioautography and analyzed. Turnover numbers for CYP3A4*1 and mutants were determined by counting the radioactivity of the TLC spots.

Chlorpyrifos Metabolism. CYP3A4s (100 pmol) were reconstituted with dioleoylphosphatidylcholine (3 μ g/10 pmol P450), NADPH reductase (400 pmol), and cytochrome *b₅* (200 pmol) added in this order. The reaction was initiated by adding 100 μ M chlorpyrifos in 100 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) with the NADPH generating system (the final concentration was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). The final assay volume was 500 μ l. The 30-min incubation was terminated by the addition of 500 μ l of ice-cold acetonitrile and vortexing. After 5 min of centrifugation at 15,000 rpm, the supernatant was analyzed for chlorpyrifos-oxon and trichloropyridinol concentrations by HPLC. The HPLC system used in this study consisted of two Shimadzu pumps (LC-10AT; Kyoto, Japan) and a Shimadzu auto injector (SIL-10AD VP). The mobile phase for pump A was 10% acetonitrile, 89% water, and 1% phosphoric acid, whereas for pump B it was 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min.

Metabolites were separated by a C₁₂ column (Synergi Max 4 μ , 150 \times 4.6 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm by a Waters 486 tunable absorbance detector (Milford, MA). Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve.

Statistical Analysis. All enzymatic data were analyzed by analysis of variance followed by Student's *t* test. *N* is the number of samples used in study. Differences were considered significant at *P* < 0.05.

Molecular Modeling. A molecular model was developed for the human CYP 3A4 wild-type protein using the technique of comparative/homology modeling. The polymorphism residue side chains were identified and modified in the completed wild-type model. The template structure used for development of the homology model was the solved mammalian microsomal rabbit cytochrome P450 2C5/2C3 chimeric structure (protein database entry: 1DT6) (Williams et al., 2000). The Molecular Simulations homology modeling package was used in a manual mode for development of the homology model. All molecular dynamics studies of the protein were performed using the Discover, Lifson and Hagler, Consistent Valence Force Field. The model was developed based on a multiple sequence alignment (Fig. 4) of the solved crystal structure (protein database: 1DT6) sequence with human cytochrome P450 2C8, 2C9, 2C18, 2C19, and 3A4 sequences. The multiple sequence alignment was performed manually based on the published P450 alignments of Gotoh (1992; Lewis, 1998) and D. Nelson (<http://drnelson.utmem.edu>). The model required insertion of seven small loops ranging from three to nine residues inserted using the loop generation program present within the Molecular Simulations homology program. There were no deletions. Discontinuities, steric bumps, and overlaps were resolved with molecular dynamics.

Results

Direct Sequencing. Genomic sequencing of all exons and intron-exon junctions was performed on DNA from 72 different human lymphoblastoid cell lines selected from individuals of varied racial and ethnic ancestries [24 individuals with African ancestry (16 African-Americans and 8 African pygmies), 24 Asians (5 Indo-Pakistani, 5 native Taiwanese, 5 mainland Chinese, 3 Cambodians, 3 Japanese, 3 Melanesian) and 24 Caucasians (10 from Utah in the United States, 5 Druze (Lebanon), 5 eastern Europeans, and 5 Russians)]. Twenty-eight SNPs were identified in these regions of CYP3A4 (Table 1). Eight SNPs were located in the exonic regions: R162Q, F189S, I193I, L293P, A297A, T346T, M445T, and P467S. The remaining SNPs were distributed in the 5'-upstream, introns, and 3'-flanking region. Sequencing results showed that R162Q was only detected in Black populations with an allelic frequency of 4% (African-Americans 7.1%, pygmies 0%). F189S was detected only in Caucasians with an allelic frequency of 2% (ethnic frequencies 10% in Eastern Europeans, not found in other Caucasian groups). Two SNPs were only found in Asians. L293 was found in Asians with a frequency of 2% (ethnic frequencies of 10% in Chinese, 0% in other Asian groups). P467S was also found in Asians with an allelic frequency of 2% (ethnic frequencies were 12% in Indo-Pakistani and 0% in other Asian ethnic groups). M445T was only detected in Caucasians in our study with an allelic frequency of 4% (Eastern Europeans: frequency of 10%, Caucasians from Utah 5.6%, not detected in other Caucasian ethnic groups). None of the samples was homozygous for the coding SNPs. No new putative splice sites were introduced by any of these coding, noncoding, or intron SNPs.

TABLE 1
CYP3A4 SNPs^a

SNPs	Site	Amino Acid Substitution	Nucleotide Substitution	Allelic Frequencies			Location in CYP3A4 Genome
				Africans 24	Caucasians 24	Asians 24	
				%			
1	5'-UTR		aatcc(A/G)acagc	0	0	2.1	148874
2	Intron 2		ttca(T/C)tggt	14.6	0	0	154915
3	Intron 3		agctc(T/A)tgca	0	0	2.1	155157
4	Intron 4		aactg(A/T)gttag	0	0	2.1	162802
5	Intron 5		tggtg(T/G)tggt	0	0	2.1	163198
6	Intron 6		ccagc(T/G)gcctg	0	4.2	0	163355
7	Exon 6	R162Q	gaggc(G/A)ggaag	4.2	0	0	163267
8	Intron 7		atctt(T/G)ctctc	50.0	2.1	2.2	164751
9	Intron 7		tgaga(T/C)ataaa	8.7	0	0	164781
10	Intron 7		attca(T/G)ccact	2.2	0	0	164802
11	Intron 7		tgtag(T/C)acatt	6.5	0	0	164835
12	Exon 7	F189S	atcat(T/C)tgag	0	2.1	0	164613
13	Exon 7	Silent	aacat(C/T)gactc	4.2	0	0	164626
14	Intron 9		gacac(AT/-)gtttg ^b	0	0	4.2	166813
15	Intron 10		ggatg(G/A)tacat	73	14.6	37.5	169228
16	Intron 10		cttag(C/T)aaaaa	8.3	0	0	169263
17	Intron 10		aaaaa(G/C)cataa	10.4	0	0	169307
18	Intron 10		gttct(G/A)ttctt	2.1	0	0	170793
19	Exon 10	L293P	cgatc(T/C)ggagc	0	0	2.1	169068
20	Exon 10	Silent	gtggc(C/T)caatc	2.1	0	0	169081
21	Intron 11		aagaa(A/G)cccta	0	2.1	0	172022
22	Intron 11		accaa(C/T)gtgga	21	0	2.1	172079
23	Exon 11	Silent	cccac(C/A)tatga	0	0	2.1	170821
24	Exon 12	M445T	tggca(T/C)gaggt	0	4.2	0	172170
25	Exon 12	P467S	tcaaa(C/T)cttgt	0	0	2.1	172235
26	3'-UTR		aaata(A/T)ccggg	4.2	0	0	175065
27	3'-UTR		gtaca(T/G)gcatt	2.1	0	0	175082
28	3'-UTR		ctgca(C/T)attaa	2.1	0	0	175416

UTR, untranslated region.

^a Accession number of CYP3A4 genomic DNA in GenBank is NG_000004.^b Deletion 2 bases (AT).

Expression and Enzymatic Assay of CYP3A4 Recombinant Alleles. The CYP3A4 alleles were inserted into the pCW expression vector, expressed in *E. coli*, and partially purified with the exception of the newly discovered R162Q allele, which we are currently attempting to express. Comparison of Western blotting (data not shown) and CO spectra of the mutants indicated that all were present as the holoprotein. Both CYP3A4*1 and all mutant CYP3A4s metabolized radioactive testosterone into 6 β -OH testosterone as the only detectable metabolite (Fig. 1). CYP3A4-P189S exhibited a lower turnover number (1.9 nmol/min/nmol) than CYP3A4*1 (7.03 nmol/min/nmol) ($P < 0.05$). Conversely, CYP3A4-L293P metabolized testosterone at a higher rate

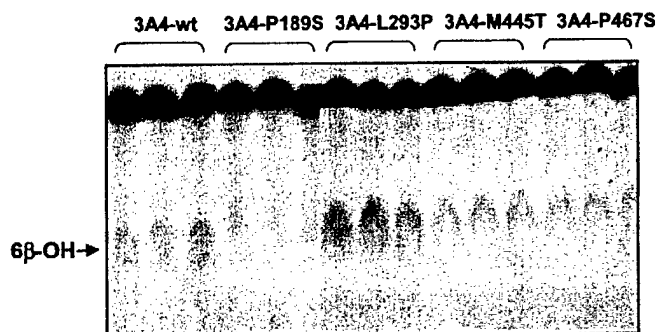


Fig. 1. Metabolism of testosterone by CYP3A4s. TLC profile of testosterone hydroxylation assay. Purified recombinant CYP3A4 wild-type and mutant proteins (10 pmol) were reconstituted and incubated with ¹⁴C-testosterone (25 μ M) for 5 min as described under *Materials and Methods*. Each assay was run in triplicate. 6 β -OH testosterone was the only metabolite detected in significant amounts.

(12.4 nmol/min/nmol) than CYP3A4*1 ($P < 0.05$) (Fig. 2). The turnover numbers for M445T and P457S were 5.8 and 5.9 nmol/min/nmol, respectively.

Chlorpyrifos Metabolism. The active metabolite chlorpyrifos-oxon and the inactive product trichloropyridinol were the major metabolites of chlorpyrifos by CYP3A4 (Figs. 3 and 4). The mutant allele L293P exhibited an increased turnover number for both metabolites ($P < 0.01$). In contrast, the F189S allele exhibited a lower turnover number for formation of both CPO

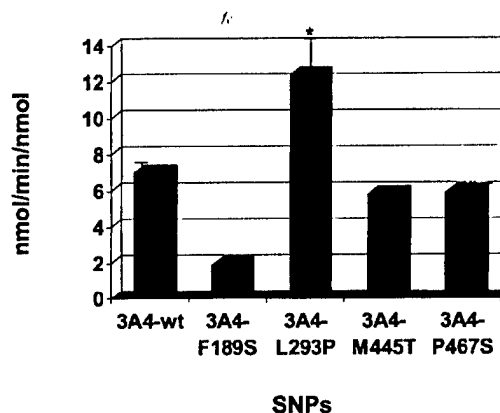


Fig. 2. Metabolism of testosterone. 6 β -OH-testosterone is the principle metabolite produced by CYP3A4*1 and its allelic variants. Purified recombinant CYP3A4 wild-type and mutant proteins (10 pmol) were reconstituted and incubated with testosterone (25 μ M) for 5 min as described under *Materials and Methods*. The turnover numbers for CYP3A4*1, F189S, L293P, M445T, and P457S are 7.03, 1.9 ($P < 0.05$), 12.4 ($P < 0.05$), 5.8, and 5.9 nmol/min/nmol, respectively.

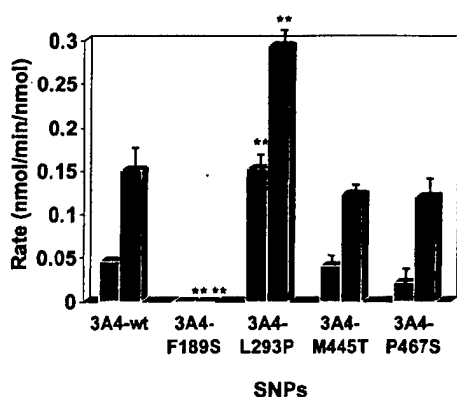


Fig. 3. Metabolism of chlorpyrifos CYP3A4 metabolizes chlorpyrifos into TCP (□) and CPO (■). CYP3A4s (100 pmol) were reconstituted and were then incubated with 100 μ M chlorpyrifos in 100 mM potassium phosphate buffer with 3.3 mM $MgCl_2$ (pH 7.4) with the NADPH generating system (the final concentration was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) for 30 min. The metabolites of chlorpyrifos-oxon (CPO) and trichloropyridinol (TCP) were analyzed by HPLC.

and TCP. The remaining two alleles did not significantly change the turnover numbers for either metabolite.

Model of CYP3A4. CYP3A4 was aligned with rabbit CYP2C5. Figure 5 shows the results of modeling CYP3A4 based on the known crystal structure of CYP2C5 (protein database entry: 1DT6). Mutations are indicated in blue. The heme is indicated in purple and the substrate testosterone in gray. The identified variants are predicted from the model to be located near the following identified structural features; R162Q is located at the end of helix D, F189S at the end of helix E, L293P is at the beginning of helix I, M445T at the beginning at helix L and P467S in a β -sheet near the C terminus of the protein. The M445T SNP is located on the other side of the heme from the ligand binding site and helix I. Side chains of residues F189, L292, and P467 are largely buried and packed into the interior of the protein. Side chains of residues R162 and M445 are largely surface exposed.

Discussion

CYP3A4 is known to metabolize many clinically important drugs, such as rifampicin, cyclosporine, and ritonavir (Kolars et al., 1992; Boxenbaum, 1999; Guengerich, 1999; Hesse et al., 2001) as well as endogenous compounds such as testosterone (Wang et al., 1997). The distribution of metabolism of CYP3A substrates is unimodal but metabolism of these sub-

strates shows at least a 10-fold variability in vivo (Thummel et al., 1996). Some variability may be due to the inducibility of the CYP3A4 gene by drugs and environmental chemicals. However, some of this variability is believed to be due to genetic factors. A previous study found a S222P allele in Finnish Caucasians and a M445T allele in Chinese (Sata et al., 2000). A very recent study identified seven new polymorphisms in European Caucasians (Eiselt et al., 2001). Two of these alleles, S222P and L373F, have been reported to affect catalytic activities toward certain substrates (Sata et al., 2000; Eiselt et al., 2001).

In the present study, we sequenced the coding regions and intron-exon junctions of CYP3A4 in DNAs from three different racial groups. Each racial group had been selected to represent ethnic diversity. A total of 28 SNPs in CYP3A4 were detected. Five SNPs produced amino acid substitutions including R162Q in exon 6; F189S in exon 7; L293P in exon 10; M445T in exon 12; and P467S in exon 12. Four of these are newly described alleles. Only the M445T allele had previously been reported (Sata et al., 2000; Eiselt et al., 2001). P189S and M445T were detected only in Caucasians in our study with frequencies of 2% and 4%, respectively. However, M445T has also been reported in Asians (Sata et al., 2000), indicating it is of ancient ancestry. The L293P and P467S alleles were found only in Asians, both at frequencies of 2% (L293 occurred in Chinese with a frequency of 10% while P467S occurred in Indo-Pakistani with a frequency of 12.5%). The coding change R162Q occurred only in African-Americans. In both individuals, this SNP was associated with an SNP in intron 10 (bp 169228) of the gene, in intron 7 (bp 164751), and in intron 11 (bp 172079). However, these intron SNPs were more frequent than the R162Q SNP in Africans. Interestingly, all alleles in African pygmies, and the majority (19/28) of alleles in African-Americans carried the SNP in intron 10 (bp 169228), which was not frequent in Caucasians. This SNP was also frequent in Asians (37.5%), indicating that it is associated with an ancient allele. The intron 7 SNP was also frequent in Africans (50% of the samples).

Testosterone and the OP insecticide chlorpyrifos were used as two examples of substrates for CYP3A4 to test effects of the coding mutations on function. The CYP3A4 active site is large (He et al., 1997), which explains its ability to metabolize a wide group of structurally diverse pharmacores (Ekins et al., 1999). Testosterone was selected as an example of a spatially large molecule that is metabolized by CYP3A4. Coding polymorphisms might potentially affect orientation of large substrates preferentially over their effects on binding of smaller substrates. Interestingly, the F189S and L293P mutations affected metabolism of both substrates in a similar fashion. The F189S allele exhibited significantly lower turnover numbers for both testosterone and chlorpyrifos than wild-type CYP3A4, while the L293P allele exhibited higher turnover numbers for both substrates. These results indicated that individuals might potentially have alterations in their ability to metabolize not only testosterone but potentially other pharmacores. Future studies will address metabolism of clinically important drugs.

Based on comparisons of the model of CYP3A4 with the crystal structure of CYP2C5, we would predict that the mutation at L293P is at the beginning of the I helix while residue F189S is at the end of helix E. These residues are not predicted to reside in the active-site cavity where they would

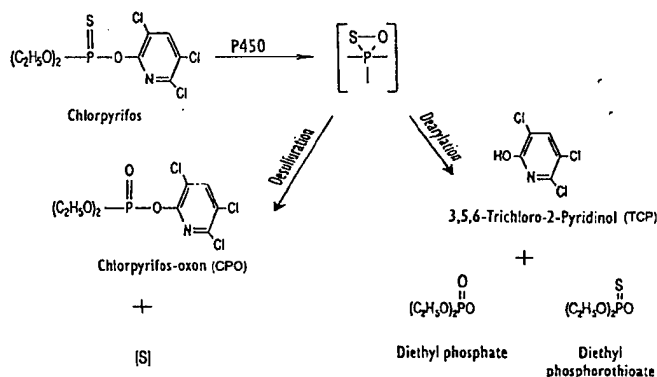


Fig. 4. Metabolic pathway of chlorpyrifos by P450.

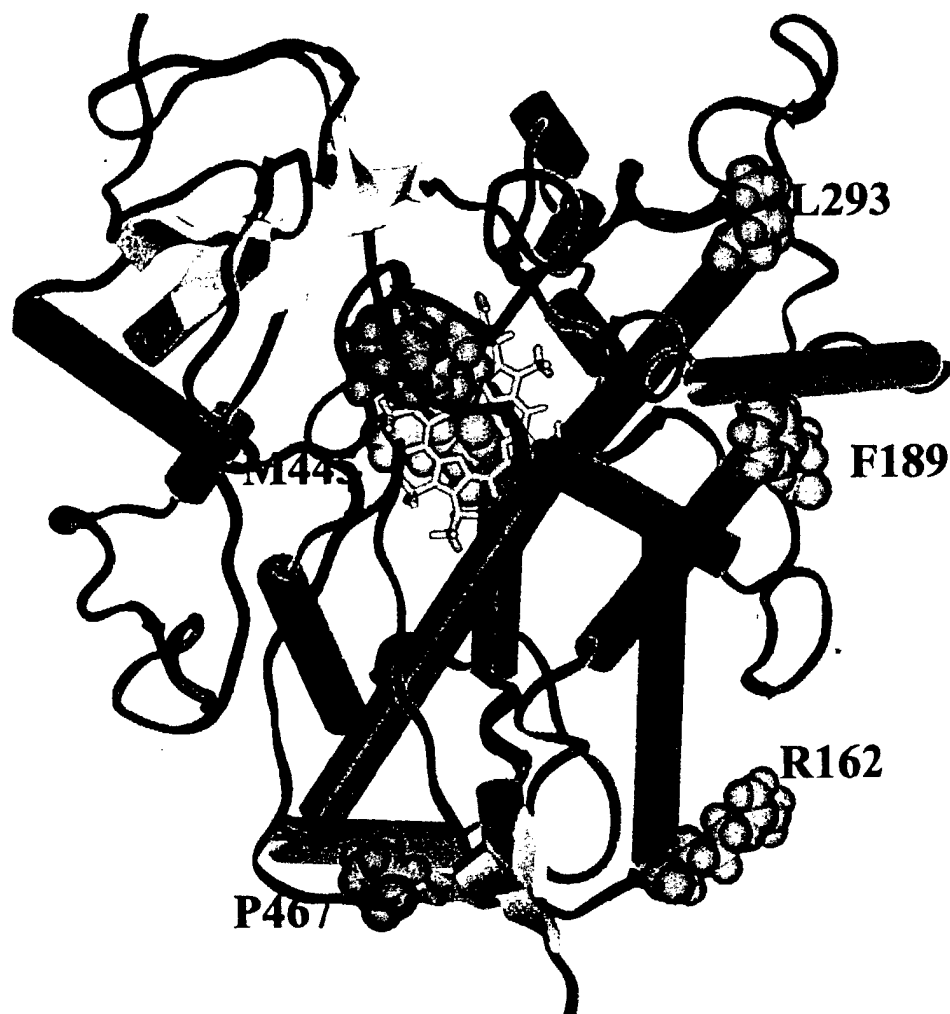


Fig. 5. Crystal structure of CYP3A4 showing locations of amino acid variants. Ribbon diagram of the molecular model of the human cytochrome P450 3A4 based on the solved x-ray crystal structure of the rabbit microsomal cytochrome P450 2C5 (protein database entry: 1DT6) (Williams et al., 2000). Complete side chains are shown in blue for the identified SNPs. The substrate testosterone is shown in gray docked in the enzyme active site. The heme is shown in purple.

directly interact with the substrate. However, the residues are nonconservative mutations in tightly packed regions, which could conceivably affect the conformation of the protein, substrate access, and/or catalytic activity. Our results indicating that the M445T mutation has no effect on testosterone or chlorpyrifos metabolism are consistent with the very recent report by Eiselt et al. (2001) using testosterone and progesterone as substrates. Modeling based on the crystal structure of CYP2C5 predicts that the M445T SNP is located in close proximity to the heme, but it is on the opposite side of the heme from the ligand binding site on helix I. This is consistent with the data indicating that this mutation may not affect catalytic activity.

CYP3A4 is important in the metabolism of environmental compounds as well as clinically important drugs. CYP3A4 is known to activate the OP insecticides parathion and chlorpyrifos into oxons that are neurotoxicants (Butler and Murray, 1997; Tang et al., 2001) (Fig. 5). CYP3A4 also inactivates chlorpyrifos into 2,3,5-trichloro-2-pyridinol. The relative rates of activation and inactivation are critical to the toxicity of the compound. The L293P allele could possibly increase

the toxicity of OP insecticides to individuals carrying this allele. Interestingly, the F189S allele decreased both activation and inactivation of chlorpyrifos and could also potentially affect toxicity after exposure to OP insecticides. In addition, CYP3A4 can activate aflatoxin B₁ into the reactive form, aflatoxin B₁-8, 9-epoxide, which is a mutagen (Gallagher et al., 1996; Chen et al., 1998). Thus polymorphisms of CYP3A4 could potentially influence the risk of different populations from various environmental compounds.

In summary, five coding polymorphisms in *CYP3A4* were identified as M445T (*CYP3A4*3*), R162Q(*CYP3A4*15*), F189S (*CYP3A4*16*), L293P(*CYP3A4*17*), and P467S (*CYP3A4*18*) by resequencing 72 individuals from three diverse racial groups.¹ Four of these represent newly described *CYP3A4* alleles. Two SNPs occurred in Caucasians (F189S

¹New CYP3A4 alleles were submitted to the CYP allele web page (www.imm.ki.se/CYPalleles). The names designated by the international nomenclature committee are *CYP3A4*17* (F189S) and *CYP3A4*18* (L292P and *CYP23A4*19* (P467S)). R162Q was submitted to the CYP3A4 allele web page by another laboratory while this work was in progress and was designated *CYP3A4*15* but has not yet been published otherwise.

and M445T), while two occurred in Asians (L293P and P467S). M445T has also been reported previously in Asians. One coding SNP R162Q was detected only in African-Americans. Testosterone and the OP insecticide chlorpyrifos were selected to assess their catalytic activities of four new alleles. The F189L allele exhibited significantly a lower turnover number for both substrates than CYP3A4*1, while the L293P allele metabolized both substrates with a higher turnover number. Potentially, these alleles may contribute to the known variability in metabolism of clinically used drugs and environmental compounds that are CYP3A4 substrates. Future studies will examine a wider range of CYP3A4 substrates including clinically used drugs.

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***IN VITRO* HUMAN METABOLISM AND INTERACTIONS OF THE REPELLENT,
N,N-DIETHYL-*M*-TOLUAMIDE (DEET).**

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A list of non-standard abbreviations:

DEET - *N,N*-diethyl-*m*-toluamide

BALC - *N,N*-diethyl-*m*-hydroxymethylbenzamide

ET - *N*-ethyl-*m*-toluamide

HLM - human liver microsomes

MLM - mouse liver microsomes

RLM - rat liver microsomes

CYP - cytochrome P450

HPLC - high performance liquid chromatography

ABSTRACT

Oxidative metabolism of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) by pooled human liver microsomes (HLM), rat liver microsomes (RLM), and mouse liver microsomes (MLM) was investigated. DEET is metabolized by cytochrome P450s (CYP) leading to the production of a ring methyl oxidation product, *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) and an *N*-deethylated product, *N*-ethyl-*m*-toluamide (ET). Both the affinities and intrinsic clearance of HLM for ring hydroxylation are greater than those for *N*-deethylation. Pooled HLM show significantly lower affinities (K_m) than RLM for metabolism of DEET to either of the primary metabolites (BALC and ET). Among 15 cDNA-expressed CYP enzymes examined, CYP1A2, 2B6, 2D6*1 (Val₃₇₄), and 2E1 metabolized DEET to the BALC metabolite while CYP3A4, 3A5, 2A6, and 2C19 produced the ET metabolite. CYP2B6 is the principal cytochrome P450 involved in the metabolism of DEET to its major BALC metabolite while CYP2C19 had the greatest activity for the formation of the ET metabolite. Use of phenotyped HLM demonstrated that individuals with high levels of CYP2B6, 3A4, 2C19, and 2A6 have the greatest potential to metabolize DEET. Mice treated with DEET demonstrated induced levels of the CYP2B family, increased hydroxylation, and a 2.4 fold increase in the metabolism of chlorpyrifos to chlorpyrifos-oxon, a potent anticholinesterase. Preincubation of human CYP2B6 with chlorpyrifos completely inhibited the metabolism of DEET. Preincubation of human or rodent microsomes with chlorpyrifos, permethrin, and pyridostigmine bromide, alone or in combination, can lead to either stimulation or inhibition of DEET metabolism.

chemical to inhibit and/or induce the metabolism of another chemical. For example, the phosphorothioate organophosphate pesticides, chlorpyrifos, is capable of inhibiting metabolism of other chemicals due to its ability to irreversibly inhibit metabolizing enzymes such as cytochrome P450s (CYPs) (Butler and Murray, 1997). Thus an understanding of how DEET is metabolized in humans, the isoforms responsible for such metabolism, and the possible interactions of DEET in metabolism of other chemicals will aid in the evaluation of the possible role which DEET may play in deployment related illnesses.

The available literature on the pharmacokinetics and metabolism of DEET has been reviewed (Qui et al., 1998). In rat, oxidative metabolism appears to account for most, if not all, of the metabolites derived from DEET. The major metabolites are *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) and *N*-ethyl-*m*-toluamide (ET) (Fig. 1), indicating that either the *N*-ethyl or the ring methyl groups can be oxidized (Taylor, 1986; Yeung and Taylor, 1988; Taylor and Spooner, 1990, Schoenig et al., 1996; Constantino and Iley, 1999). These two major and several minor metabolites were characterized in liver microsomes from phenobarbital-pretreated rats (Taylor, 1986; Yeung and Taylor, 1988; Constantino and Iley, 1999). Studies of the metabolism of DEET by human enzymes are lacking. Studies of absorption and metabolism in humans have been conducted only at the level of determining urinary metabolites and suggest that 5-8 percent of topically applied DEET is rapidly absorbed and excreted. As many as six metabolites were recovered from the human urine samples (Selim et al., 1995).

The main objectives of the present study were to identify and quantify the oxidative metabolism of DEET by human liver microsomes and to compare the metabolism of DEET in

human liver microsomes with that in rat and mouse liver microsomes. Further objectives were to identify the human CYP isoforms responsible for DEET metabolism, and to investigate potential interactions of DEET with other xenobiotics.

Materials and Methods

Chemicals. DEET, chlorpyrifos, chlorpyrifos-oxon, 3,5,6-trichloro-2-pyridinol, and permethrin (50:50 *cis-trans*) were purchased from ChemService (West Chester, PA). *N*-Ethyl-*m*-toluamide (ET) and *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) were synthesized as described previously (Taylor, 1986). Pyridostigmine bromide was purchased from Roche (Indianapolis, IN). HPLC grade acetonitrile and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA) and Mallinckrodt Baker, Inc. (Paris, Kentucky), respectively. All other chemicals were purchased, if not specified, from Sigma (St. Louis, MO).

Rodent liver microsome preparation. Rat liver microsomes (RLM) and mouse liver microsomes (MLM) were prepared from adult male Long Evans rats and adult male CD-1 mice (Charles River Laboratories, Raleigh, NC), respectively, according to the method of Cook and Hodgson (1983). Briefly, immediately after sacrificing the animals, the fresh livers were excised, weighed, minced, and washed with ice cold homogenized buffer (50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 1.15% potassium chloride). Samples were homogenized with a Polytron homogenizer in ice-cold homogenization buffer and centrifuged at 10,000 *g* for 15 minutes. The supernatant was filtered through glass wool, centrifuged at 100,000 *g* for 1 hour, and the microsomal pellet was resuspended in storage buffer (50 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 0.25 M sucrose). All processes were performed at 0-4°C. The microsomal preparation was aliquoted and stored at -80°C until used.

Total cytochrome P450 content was determined by the CO-difference spectrum method of Omura and Sato (1964). Protein concentration was determined using the BioRad protein assay with

bovine serum albumin (BSA) as standard (Bradford, 1976).

Human liver microsomes and human cytochrome P450 isoforms. Pooled human liver microsomes (HLM) (pooled from 10 donors) and human cytochrome P450 (CYP) isoforms expressed in baculovirus infected insect cells (Sf9) (BTI-TN-5B1-4), CYP1A1, 1A2, 2A6, 1B1, 2B6, 2C8, 2C9*1 (Arg₁₁₄), 2C18, 2C19, 2D6*1 (Val₃₇₄), 2E1, 3A4, 3A5, 3A7, and 4A11 were purchased from Gentest Corporation (Woburn, MA). Gender specific HLM (pooled from 5 male donors or 5 female donors, respectively) were purchased from Gentest Corporation (Woburn, MA). Gender specific HLM (pooled from 10 male donors or 10 female donors, respectively) were also purchased from Xenotech, LLC (Kansas City, KS). Selected individual human liver microsomes (HG042, HG043, and HG095) were purchased from Gentest Corporation (Woburn, MA).

***In vitro* DEET metabolism.** Enzyme kinetic assays for microsomes were performed by incubation in 1.5 ml microcentrifuge tubes of serial concentrations of DEET (final concentrations 31.24 - 3000 μ M) with microsomes in 20 mM Tris-HCl buffer (pH 8.3 at 37°C) containing 5 mM MgCl₂ (final volume 1.0 ml) for 5 min. Preliminary studies demonstrated that a pH of 8.3 was optimal for the production of DEET metabolites as had previously been reported for metabolites of DEET by rat liver microsomes (Yeung and Taylor, 1988). The microsomal protein concentrations used in assays were 1.5 mg/ml for HLM, RLM, and MLM. After preincubation at 37°C for 5 min, reactions were initiated by the addition of ice cold microsomes to prewarmed buffer/substrate/cofactors. The final concentration of the NADPH generating system was 0.25 mM NADP, 2.5 mM glucose-6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase. The controls were performed in the absence of the NADPH generation system. Reactions were terminated by the

addition of an equal volume of acetonitrile and vortexing. After 10 min centrifugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for BALC and ET concentrations by HPLC as described below.

The initial metabolic activity assays for human cytochrome P450 (CYP) isoforms were performed by incubation of DEET (final concentrations 1000 or 3000 μM) with CYP isoforms (final P450 contents 100 - 200 pmole/ml) for 20 min in CYP-specific buffers recommended by the supplier (Gentest, Woburn, MA). The controls were performed in the absence of an NADPH generation system. For CYP1A1, 1A2, 1B1, 2D6*1 (Val₃₇₄), 3A4, 3A5, and 3A7 a 100 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For CYP2B6, 2C8, 2C19, and 2E1 a 50 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For CYP2C9*1 (Arg₁₄₄), 2C18, and 4A11 a 100 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.5) was used. For CYP2A6 a 50 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.5) was used.

Enzyme kinetic assays for human CYP1A2, 2B6, and 2D6*1 (Val₃₇₄) were performed by incubations of serial concentrations of DEET (final concentrations 31.25 - 1000 μM) (final P450 content 50 pmole) for 10 min in CYP-specific buffers recommended by the supplier (Gentest, Woburn, MA).

Assays of five pooled male and female HLM purchased from Gentest and ten pooled male and female HLM purchased from Xenotech were performed by incubations of DEET (final concentration 1000 μM) with microsomes (final protein concentration 1.5 mg/ml) for 10 min. Similar conditions were used for assays of individual HLM.

Induction. Adult male CD-1 mice, 28-30 grams, were obtained from Charles River

Laboratories and acclimated for 4 days. Low (2 mg/kg/day), medium (20 mg/kg/day), and high (200 mg/kg/day) doses of DEET in 100 μ l corn oil were given intraperitoneally daily for 3 days. The dose range for DEET was selected in order to not exceed a dose known to produce a physiological effect (Chaney et al., 1999). Doses approximating LD₁₀ values for phenobarbital (80 mg/kg/day) in 100 μ l water or 3-methylcholanthrene (20 mg/kg/day) in 100 μ l corn oil were also administered intraperitoneally, to separate groups of mice, daily for 3 days. Controls were given corn oil only or water. Microsomes were prepared from livers of fed mice on the fourth day as described above.

The following substrates were used as indicators of the activities for the following isozymes: ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) for CYP1A1/2 (Burke and Mayer, 1974; Nerurkar et al., 1993), pentoxyresorufin O-dealkylation (PROD) for CYP2B10 (Lubet et al., 1985) and benzyloxyresorufin O-dealkylation (BROD) for CYP2B (Nerurkar et al., 1993). Assays were conducted as described by Pohl and Fouts (1980). Briefly, assays were initiated by the addition of an NADPH regenerating system and incubated for 5 minutes at 37°C. Product formation for EROD, MROD, PROD, and BROD activities were determined spectrofluorimetrically (F-2000 Fluorescence spectrophotometer, Hitachi Ltd. Tokyo, Japan) by comparison with a standard curve generated with resorufin.

Chlorpyrifos metabolism. Chlorpyrifos metabolism activity assays were performed by incubation of 100 μ M chlorpyrifos with induced MLM for 5 min. The microsomal protein concentrations used in the assays were 1.0 mg/ml in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 5 mM MgCl₂ and 3 mM EDTA. Reactions were stopped by the addition of acetonitrile followed by centrifugation at 15,000 rpm. The supernatant was analyzed for chlorpyrifos-oxon and

3,5,6-trichloro-2-pyridinol concentrations by HPLC as described by Tang et al. (2001).

Inhibition. The inhibition of human CYP2B6 by chlorpyrifos and chlorpyrifos-oxon were studied by incubating these two compounds (final concentration 100 μ M), CYP2B6 (50 pmoles), NADPH generating system, and 50 mM potassium phosphate buffer with 3.3 mM $MgCl_2$ (pH 7.4) for 5 min at 37°C prior to adding DEET (final concentration 1000 μ M). The reaction was terminated after an additional 20 min by the addition of an equal volume of acetonitrile. After brief centrifugation the supernatant was analyzed for BALC concentration by HPLC.

In order to understand possible interactions produced by potential induction of metabolizing enzymes by DEET; effects on DEET metabolism were examined in HLM, MLM and RLM following preincubations with chlorpyrifos, permethrin, and pyridostigmine bromide either alone or in combination (final concentration 100 μ M). In addition, the effects of these compounds were also examined in some mice which had been induced with phenobarbital (80 mg/kg/day), 3-methylcholanthrene (20 mg/kg/day) or the high dose of DEET (200 mg/kg/day). Microsomes (final protein concentrations 1.5 mg/ml), NADPH generating system, and 20 mM Tris-HCl buffer with 5 mM $MgCl_2$ (pH 8.3 at 37°C) were incubated for 5 min at 37°C prior to adding DEET (final concentration 1000 μ M). The reaction was terminated after 10 min by the addition of an equal volume of acetonitrile. Following centrifugation at 15,000 rpm, the supernatant was analyzed for BALC and ET concentrations by HPLC.

All assays were conducted in triplicate. The protein concentrations and incubation times used in the assays were found to be in the linear range in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH generating system.

Analysis of metabolites by HPLC. Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan). The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of 2 pumps (LC-10AT), a Shimadzu auto injector (SIL-10AD *VP*), and a Waters 486 Tunable Absorbance Detector (Milford, MA). For DEET metabolites, the mobile phase for pump A was 3.5% tetrahydrofuran, 96.5% water, for pump B 100% acetonitrile. A gradient system was employed as described by Yeung and Taylor (1988) in the following manner: 0-3 min (10% B), 3-30 min (10-60% B), 30-32 min (60-10% B), and 32-35 min (10% B). The flow rate was 1 ml/min. For chlorpyrifos metabolites, the mobile phase for pump A was 10% acetonitrile, 89% H₂O and 1% phosphoric acid, for pump B 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min. Metabolites for DEET and chlorpyrifos were separated by a C18 column (Luna 5 μ , 150 X 3 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm. Under these conditions the retention times for BALC, ET, and DEET were 2.5, 5, and 11 min, respectively. The limit of detection for the two DEET metabolites was approximately 10 μ M. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. K_m and V_{max} were obtained from Lineweaver-Burk plots (1/velocity vs 1/substrate concentration).

Statistics. Significant differences between data sets were determined by one way analysis of variance and multiple comparisons were performed with the Tukey-Kramer honestly significant different (HSD) test using a SAS program (SAS, 1989).

Results

Pooled HLM as well as RLM and MLM showed a much lower K_m (higher affinity) and higher intrinsic clearance for ring hydroxylation (BALC formation) than for *N*-deethylation (ET formation) from DEET (c. 10-fold differences) (Table 1). HLM also exhibited higher K_m s (lower affinities) than either RLM or MLM. HLM exhibited a lower intrinsic clearance rate [CL_{int} (V_{max}/K_m)] for both metabolites than RLM. However, the CL_{int} for MLM were similar to those of HLM. When mice were treated with the high dose (200 mg/kg/day) there was a significant increase in the V_{max} and intrinsic clearance of BALC and ET indicating that DEET induced its own metabolism.

Among 15 different human CYP isoforms screened, only CYP1A2, 2B6, 2D6*1 (Val₃₇₄), and 2E1 displayed detectable BALC metabolite production (Table 2). The activity of CYP2E1 was significantly less than the activities of the other CYP's producing the BALC metabolite. Production of the BALC metabolite was generally much higher than that of the ET metabolite. Isoforms producing detectable amounts of the ET metabolite included CYP3A4, 3A5, 2A6, and 2C19. These isoforms produced no detectable amounts of the BALC metabolite. CYP2C19 showed significantly higher activity than CYP3A4, 3A5, and 2A6. The following isoforms, CYP1A1, 1B1, 3A7, 2C8, 2C9*1 (Arg₁₄₄), 2C18, and 4A11, were inactive in the production of either metabolite.

Kinetic studies of CYP1A2, 2B6, and 2D6*1 (Val₃₇₄) with respect to the production of the ring methyl oxidation product BALC are shown in Table 3. No significant differences in K_m , V_{max} , and CL_{int} (V_{max}/K_m) in production of the BALC metabolite were observed between CYP1A2 and 2B6. Activity of the CYP2D6 isoform was too low for accurate kinetic determinations. Comparisons of

male and female differences in metabolism of DEET were performed using pooled liver microsomes from two different suppliers. Microsomes from Gentest included five pooled males and females while those from Xenotech included ten individuals from each gender. In both cases, activity of females in the production of BALC and ET metabolites was greater than that of males, however, due to departure from randomness in the way these pooled samples were prepared the data are insufficient to demonstrate a definitive gender difference (data not shown).

To further determine the importance of CYP2B6 and 1A2 in ring methyl oxidation of DEET and, in addition, the importance of CYP3A4, 2C19, and 2A6 in *N*-deethylation of DEET, liver microsomes from three different individuals possessing varying levels of these isoforms were investigated with respect to their ability to metabolize DEET (Table 4). The individual with high levels of both CYP2B6 and 1A2 (HG042) had significantly greater ability to produce the BALC metabolite than the other two individuals. In contrast, individuals with high levels of CYP1A2 (HG043) or CYP2D6 (HG095) had significantly lower ability to metabolize DEET to the BALC metabolite, indicating the importance of CYP2B6 in formation of this metabolite. The individual with high levels of CYP3A4 and 2A6 but low level of CYP2C19 (HG042) had the highest activity for production of the ET metabolite. The individual (HG043) with the highest level of CYP2C19 but low levels of CYP3A4 and 2A6 had significantly greater ability to produce the ET metabolite than the individual (HG095) with very low levels of these isoforms.

Experiments were conducted to examine the potential of DEET to induce enzymes involved in metabolism. These experiments included the prototypical CYP inducers phenobarbital and 3-methylcholanthrene. Doses of DEET were low, medium and high (2, 20 and 200 mg/kg/day). As

expected, phenobarbital and 3-MC induced CYP content. Phenobarbital induced BROD (5.2-fold) and PROD (30-fold) activities, while 3-MC induced EROD (23-fold) and MROD (10.5-fold) activities. No significant levels of induction were observed for the two lower doses of DEET. In contrast, the high dose of DEET produced significant increases in BROD (3.5-fold) and in PROD activities(4.0-fold).

Studies were also conducted to examine the possible effect of DEET, phenobarbital and 3-methylcholanthrene to induce metabolism of chlorpyrifos in mice. No significant differences were observed in the dearylation of chlorpyrifos with any treatment. However, significant increases in chlorpyrifos desulfuration activity were observed with phenobarbital (5.5-fold) and the high dose of DEET (2.8-fold).

The possibility that chlorpyrifos or chlorpyrifos-oxon may inhibit DEET metabolism by human CYP2B6 was investigated by incubating 100 μ M concentration of each substrate for 5 min prior to addition of DEET as a substrate. Chlorpyrifos preincubation resulted in 100% inhibition of the production of the BALC metabolite, while for chlorpyrifos-oxon, 58% inhibition was observed.

The effects of chlorpyrifos, permethrin, and pyridostigmine bromide, alone or in combination on DEET metabolism were investigated using human, rat, and mouse liver microsomes (Table 5). Preincubation of pooled HLM with permethrin, pyridostigmine bromide, and permethrin + pyridostigmine bromide significantly increased the production of BALC. Preincubation of pooled HLM with chlorpyrifos alone or in combination with any other compound significantly decreased the production of BALC. Preincubation of pooled HLM with chlorpyrifos, permethrin, pyridostigmine bromide, alone or in combination showed no significant differences on the

production of ET. Generally, preincubation of pooled HLM, RLM, MLM, DEET treated MLM, phenobarbital treated MLM, and 3-methylchonthrene treated MLM with chlorpyrifos alone or in combination of any other compound significantly inhibited the production of BALC and ET.

Discussion

Despite the extensive use of DEET as an insect repellent worldwide, no in vitro studies have been carried out using human enzymes and none of the rat studies have been at the level of individual isoforms. The mean metabolic intrinsic clearance rates, as estimated by V_{\max}/K_m , indicate that RLM metabolize DEET more efficiently than pooled HLM or MLM and that the BALC metabolite is produced more readily than the ET metabolite.

A previous study using rat liver microsomes demonstrated a significant gender difference in the metabolism of DEET with males having nearly 3-fold greater metabolizing ability than females (Yeung and Taylor 1988). Although some pooled female HLM showed significantly higher activities in BALC and ET production from DEET than pooled male HLM the results could not be regarded as definitive as noted above. Further research with large randomly selected pooled male and female HLM or microsomes from individuals is required to better understand possible gender differences in humans.

It is of interest that although eight different isoforms are capable of metabolizing DEET, each isoform produced only one metabolite. Results from individual incubations of DEET with various CYP isoforms suggested that CYP1A2 and 2B6 were highly active in production of the BALC metabolite, while CYP3A4, 2C19, and 2A6 were important in the formation of the ET metabolite. Inasmuch as different individuals have varying levels of each of these isoforms, we selected microsomes from individuals possessing widely varying activities of these isoforms to represent contrasting levels of predicted metabolic activity. As expected, the individual with high levels of CYP2B6 and 1A2 had the greatest ability to produce the BALC metabolite. In contrast, individuals

possessing high levels of either CYP3A4 or 2C19 had the greatest levels of the ET metabolite production while the individual with the lowest levels of these isoforms had the lowest ET metabolite production. Based on these results it seems reasonable to suggest that individuals, regardless of gender, with varying activities of these CYP isoforms, will be more or less efficient in the metabolism of DEET.

To determine which CYP isoforms were inducible by DEET, substrate specific assays were conducted using microsomes from DEET-treated mice. DEET treatment did not induce CYP1A1/1A2 as determined from EROD and MROD activity measurement. In contrast, PROD and BROD activities, measures of CYP2B isoform activity, significantly increased in the animals treated with the highest dose of DEET. These induction studies corroborated our results, which indicated that CYP2B6, similar to phenobarbital-induced CYP2B isoforms in rodents (Fabrizi *et al.*, 1999; Levi *et al.*, 1988), is one of the most important CYP isoforms in ring methyl oxidation of DEET and, furthermore, that DEET induces its own metabolism.

Tang *et al.* (2001) reported that CYP2B6 has the highest activity for desulfuration of chlorpyrifos, an activation process. In this study, mice treated intraperitoneally with the highest dose of DEET had significant induction of CYP2B. This induction was demonstrated to result in increased chlorpyrifos desulfuration (2.8-fold), resulting in the production of the activation product, chlorpyrifos oxon. The lower doses of DEET were not effective inducers of CYP2B6. Although these results may imply that DEET could increase organophosphate toxicity, the high levels necessary to produce the effects, combined with the mode of administration, might suggest that risks through epidermal exposures to humans may be minimal. Inversely, preincubation of chlorpyrifos

with CYP2B6 resulted in complete inhibition of DEET metabolism to the BALC metabolite. Thus, chlorpyrifos exposure could inhibit the subsequent metabolism of DEET in humans by inhibiting isoforms involved in DEET metabolism.

Serious side effects caused by drug-drug interactions have been reported (Guengerich, 1997). Either inhibition or induction can modulate the activity of an enzyme; P450s may exhibit stimulation (positive cooperativity) in the presence of certain xenobiotic compounds (Guengerich, 1997; Szklarz and Halpert, 1998). Our data show stimulation of pooled HLM, wherein conversion of DEET to BALC increased significantly in the presence of permethrin, pyridostigmine bromide, and permethrin + pyridostigmine bromide. However, no stimulation was observed with RLM, MLM, and MLM induced with DEET, phenobarbital, or 3-methylchonthrene in the presence of permethrin, pyridostigmine bromide, and permethrin + pyridostigmine bromide. Inhibition in a sense may be considered more serious than enzyme induction because inhibition happens quickly, not taking time to develop, as with induction (Guengerich, 1997). Our data show the inhibition of pooled HLM, RLM, MLM, and MLM induced with DEET, phenobarbital or 3-methylcholanthrene, wherein conversion of DEET to its metabolites, BALC and ET, decreased significantly in the presence of chlorpyrifos alone or in combination of any other tested compound.

In summary, the present investigation has demonstrated that human liver microsomes appear to have generally lower activities for DEET metabolism than those from rodent livers. A screen of human CYP isoforms demonstrated that different sets of isoforms are responsible for the production of each metabolite (BALC and ET). Individuals with varying levels of activities of these human CYP isoforms, regardless of gender, are more or less active in their metabolism of DEET. DEET

induction studies, conducted in mice, demonstrate that exposure could result in the induction of CYP2Bs, resulting in potential interactions with other chemicals such as chlorpyrifos. MLM from DEET treated mice metabolized chlorpyrifos more readily to chlorpyrifos-oxon, a potent anticholinesterase, than control MLM. Preincubation of chlorpyrifos alone with CYP2B6 completely inhibited the metabolism of DEET. Whereas, preincubation of microsomes with chlorpyrifos, permethrin, and pyridostigmine bromide, alone or in combinations may lead to either stimulation or inhibition of DEET metabolism.

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Unnumbered footnote:

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Table 1. Kinetic parameters for the ring methyl oxidation and *N*-deethylation of DEET by pooled human liver microsomes (HLM), rat liver microsomes (RLM), mouse liver microsomes (MLM), and DEET treated mouse liver microsomes

	BALC (Ring Methyl Oxidation)			ET (<i>N</i> -deethylation)		
	K_m μM	V_{\max} nmol/mg protein/min	CL_{int} $10^{-6}/\text{mg}$ protein/min	K_m μM	V_{\max} nmol/mg protein/min	CL_{int} $10^{-6}/\text{mg}$ protein/min
HLM	67.6 \pm 4.2 ^b	12.9 \pm 1.6 ^{ab}	191.5 \pm 15.4 ^b	842.5 \pm 49.9 ^b	20.5 \pm 3.4 ^a	24.4 \pm 5.1 ^b
RLM	38.3 \pm 0.2 ^a	17.6 \pm 1.2 ^a	461.3 \pm 23.5 ^a	214.3 \pm 26.1 ^a	19.2 \pm 2.8 ^a	89.5 \pm 10.9 ^a
MLM	43.4 \pm 0.6 ^{ab}	6.8 \pm 1.4 ^b	156.8 \pm 23.8 ^b	660.6 \pm 59.5 ^b	14.5 \pm 2.9 ^a	21.7 \pm 3.9 ^b
MLM*	42.6 \pm 13.6 ^{ab}	16.4 \pm 3.4 ^a	385.1 \pm 52.6 ^a	630.9 \pm 128.0 ^b	22.9 \pm 2.9 ^a	38.3 \pm 5.1 ^b

* DEET treated animals LD₁₀ (200 mg/kg/day).

Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean \pm SEM ($n = 3$).

Table 2. Ring methyl oxidation and *N*-deethylation activities toward DEET in human cytochrome P450 isoforms expressed in baculovirus infected insect cells

	BALC (Ring Methyl Oxidation)	ET (<i>N</i> -deethylation)
	nmol/nmol isoform/min	
1A2	68.94 ± 2.64 ^a	Not detected
2B6	69.51 ± 1.83 ^a	Not detected
2D6*1	56.56 ± 2.52 ^b	Not detected
2E1	3.34 ± 0.17 ^c	Not detected
2A6	Not detected	4.55 ± 0.30 ^a
2C19	Not detected	8.96 ± 0.82 ^b
3A4	Not detected	5.05 ± 0.20 ^a
3A5	Not detected	5.81 ± 0.24 ^a

Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean ± SEM ($n = 3$).

Table 3. Kinetic parameters for ring methyl oxidation of DEET by human cytochrome P450 isoforms expressed in baculovirus infected insect cells

	BALC (Ring Methyl Oxidation)		
	K_m	V_{max}	CL_{int}
	μM	nmol/nmol isoform/min	$10^{-6}/\text{nmol isoform/min}$
CYP2B6	40.2 ± 1.2^a	22.3 ± 2.1^a	552.0 ± 40.4^a
CYP1A2	41.0 ± 2.0^a	24.5 ± 1.2^a	598.7 ± 39.6^a
CYP2D6	> 1000		

Means in the same column followed by the same letter are not significantly different ($P < 0.01$).

Values are the mean \pm SEM ($n = 3$).

Table 4. Ring methyl oxidation and *N*-deethylation activities toward DEET in individual human liver microsomes

	BALC (Ring Methyl Oxidation) ¹	ET (<i>N</i> -deethylation) ¹
	nmol/mg protein/min	
HG042 ^d	3.30 ± 0.17 ^a	1.59 ± 0.03 ^a
HG043 ^d	0.54 ± 0.03 ^b	1.14 ± 0.05 ^b
HG095 ^d	0.52 ± 0.01 ^b	0.63 ± 0.03 ^c

¹ BALC and ET activities are based on 5 and 20 min incubation, respectively.

Means in the same column followed by the same letter are not significantly different ($P < 0.01$).

Values are the mean ± SEM ($n = 3$).

^d Individual human liver microsomes (protein concentration 20 mg/ml). CYP2B6, 2D6, 2E1, 1A2, 3A4, 2C19, and 2A6 activities (pmol product/mg protein/min), represented by (S)-mephenytoin *N*-demethylase, bufuralol 1'-hydroxylase (amount of activity inhibited by 1 μM quinidine), chlorzoxazone 6-hydroxylase, phenacetin *O*-deethylase, testosterone 6β-hydroxylase, (S)-mephenytoin 4'-hydroxylase, and Coumarin 7-hydroxylase catalytic activities, respectively, are 270, 68, 1700, 640, 13000, 8, and 2300 for HG042 (48-year-old female), 34, 20, 1100, 630, 5600, 700, and 850 for HG043 (23-year-old female), and 12, 160, 1200, 280, 760, 30, and 200 for HG095 (47-year-old female) (data were provided by Gentest, Woburn, MA).

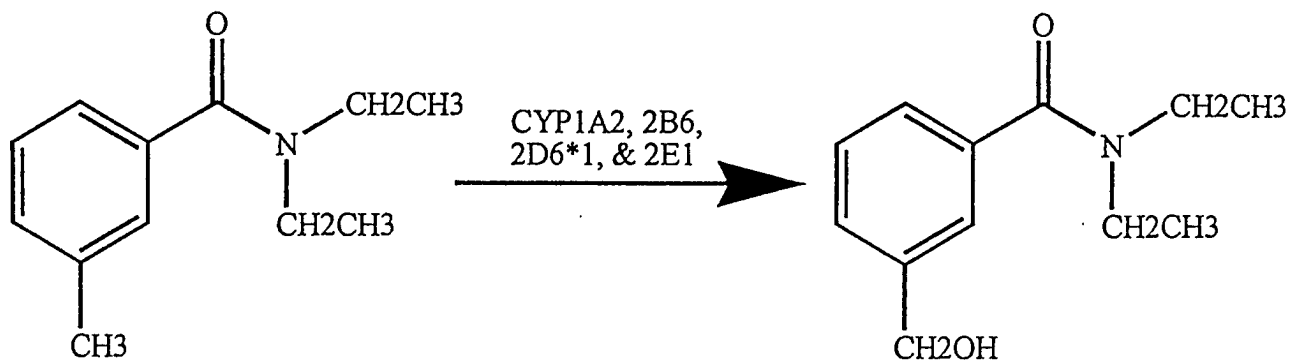
Table 5. Effects of chlorpyrifos (CPS), permethrin (PMT), pyridostigmine bromide (PYB), alone and in combination on DEET Metabolism by pooled HLM, RLM, MLM, DEET treated MLM, phenobarbital (PB) treated MLM, and 3-methylcholanthrene (3-MC) treated MLM

Treatment	HLM ^a			RLM ^a			MLM (Control) ^a			MLM (DH) ^a			MLM (PB) ^a			MLM (3-MC) ^a			
	BALC	ET		BALC	ET		BALC	ET		BALC	ET		BALC	ET		BALC	ET		
Control	1.1 ± 0.1 ^b	1.5 ± 0.1 ^a	4.3 ± 0.1 ^a	3.1 ± 0.2 ^a	1.7 ± 0.3 ^a	2.0 ± 0.2 ^a	1.9 ± 0.1 ^a	4.8 ± 0.3 ^a	4.3 ± 0.3 ^a	1.3 ± 0.1 ^a	2.0 ± 0.1 ^a	1.9 ± 0.1 ^a	4.8 ± 0.3 ^a	4.3 ± 0.3 ^a	1.3 ± 0.1 ^a	2.0 ± 0.1 ^a	1.9 ± 0.1 ^a	4.8 ± 0.3 ^a	4.3 ± 0.3 ^a
CPS	0.3 ± 0.0 ^c	1.4 ± 0.0 ^a	1.1 ± 0.1 ^c	1.6 ± 0.1 ^{bc}	0.2 ± 0.0 ^b	0.8 ± 0.2 ^b	1.0 ± 0.0 ^b	1.0 ± 0.2 ^b	1.6 ± 0.3 ^b	0.6 ± 0.1 ^b	1.0 ± 0.2 ^b	1.0 ± 0.0 ^b	1.0 ± 0.2 ^b	1.6 ± 0.3 ^b	0.6 ± 0.1 ^b	1.0 ± 0.2 ^b	1.0 ± 0.2 ^b	1.6 ± 0.3 ^b	0.6 ± 0.1 ^b
PMT	1.7 ± 0.1 ^a	1.6 ± 0.1 ^a	2.2 ± 0.0 ^b	2.3 ± 0.1 ^{ac}	0.9 ± 0.2 ^a	1.4 ± 0.2 ^a	1.8 ± 0.4 ^a	2.4 ± 0.5 ^a	3.4 ± 0.3 ^a	0.8 ± 0.1 ^{bc}	1.7 ± 0.2 ^a	2.4 ± 0.5 ^a	2.5 ± 0.2 ^c	3.4 ± 0.3 ^a	0.8 ± 0.1 ^{bc}	1.7 ± 0.2 ^a	2.4 ± 0.5 ^a	2.5 ± 0.2 ^c	3.4 ± 0.3 ^a
PYB	1.8 ± 0.1 ^a	2.1 ± 0.1 ^a	3.6 ± 0.2 ^a	3.0 ± 0.1 ^a	1.2 ± 0.2 ^a	1.8 ± 0.2 ^a	2.2 ± 0.3 ^a	2.8 ± 0.5 ^a	3.0 ± 0.2 ^a	1.2 ± 0.1 ^{ac}	1.8 ± 0.2 ^a	2.2 ± 0.3 ^a	2.9 ± 0.3 ^c	3.0 ± 0.2 ^a	1.2 ± 0.1 ^{ac}	1.8 ± 0.1 ^a	2.2 ± 0.3 ^a	2.9 ± 0.3 ^c	3.0 ± 0.2 ^a
CPS + PMT	0.6 ± 0.0 ^c	1.6 ± 0.3 ^a	1.1 ± 0.2 ^c	1.4 ± 0.0 ^{bc}	0.3 ± 0.0 ^b	0.8 ± 0.1 ^b	0.3 ± 0.0 ^b	0.9 ± 0.1 ^b	1.1 ± 0.1 ^b	0.7 ± 0.1 ^b	0.8 ± 0.1 ^b	0.3 ± 0.0 ^b	0.4 ± 0.1 ^b	1.1 ± 0.1 ^b	0.5 ± 0.1 ^b	0.7 ± 0.1 ^b	0.8 ± 0.1 ^b	1.1 ± 0.1 ^b	0.5 ± 0.1 ^b
CPS + PYB	0.4 ± 0.0 ^c	1.3 ± 0.1 ^a	1.5 ± 0.4 ^{bc}	2.0 ± 0.3 ^c	0.2 ± 0.0 ^b	0.8 ± 0.1 ^b	0.2 ± 0.1 ^b	0.8 ± 0.2 ^b	1.3 ± 0.1 ^b	0.6 ± 0.0 ^b	0.8 ± 0.1 ^b	0.2 ± 0.1 ^b	0.5 ± 0.0 ^b	1.3 ± 0.1 ^b	0.6 ± 0.0 ^b	0.9 ± 0.0 ^b	0.8 ± 0.1 ^b	1.3 ± 0.1 ^b	0.6 ± 0.0 ^b
PMT + PYB	1.6 ± 0.1 ^a	1.6 ± 0.1 ^a	2.5 ± 0.1 ^b	2.4 ± 0.2 ^{ac}	0.9 ± 0.0 ^a	1.7 ± 0.2 ^a	1.7 ± 0.5 ^a	2.4 ± 0.6 ^a	3.2 ± 0.3 ^a	0.6 ± 0.1 ^b	1.6 ± 0.1 ^a	1.7 ± 0.5 ^a	2.6 ± 0.2 ^c	3.2 ± 0.3 ^a	0.6 ± 0.1 ^b	1.1 ± 0.1 ^b	2.4 ± 0.6 ^a	2.6 ± 0.2 ^c	3.2 ± 0.3 ^a
CPS + PMT + PYB	0.6 ± 0.0 ^c	1.4 ± 0.1 ^a	0.9 ± 0.1 ^c	1.3 ± 0.0 ^{bc}	0.2 ± 0.1 ^b	0.8 ± 0.1 ^b	0.2 ± 0.0 ^b	1.0 ± 0.1 ^b	1.5 ± 0.1 ^b	0.3 ± 0.0 ^a	0.9 ± 0.2 ^b	0.9 ± 0.2 ^b	0.9 ± 0.2 ^b	1.5 ± 0.1 ^b	0.3 ± 0.0 ^a	0.9 ± 0.1 ^b	0.9 ± 0.2 ^b	1.5 ± 0.1 ^b	0.3 ± 0.0 ^a

^a nmol product formed/mg protein/min. DEET High (DH) 200 mg/kg/day, PB 80 mg/kg/day, 3-MC 20 mg/kg/day.

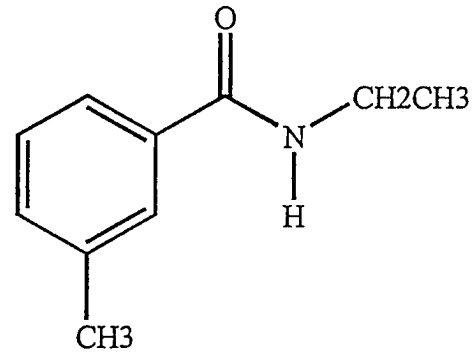
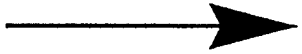
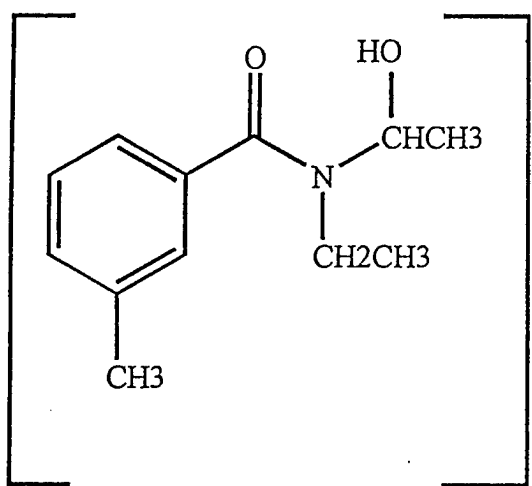
Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean ± SEM ($n = 3$).

Fig. 1. Primary in vitro metabolites of DEET



N,N-diethyl-m-toluamide (DEET)

N,N-diethyl-m-hydroxymethylbenzamide (BALC)



N-ethyl-m-toluamide (ET)